BOTULISM OF SHEEP IN WESTERN AUSTRALIA

NUTRITIONAL EXPERIMENTS I

by

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INTRODUCTION.

Although botulism in cattle has been known in Western Australia since about 1860, the disease was not recorded in sheep until 1928, when it was described by Bennetts (1933). Since 1928 its incidence has increased progressively until in the summers of 1932-3 and 1934-5 it was considered to be responsible for greater losses than all other diseases affecting sheep in Western Australia. In other parts of the world botulism is rarely the cause of serious losses in sheep, even where cases in cattle are quite frequent. It has been recorded in only one other part of Australia, namely New South Wales, where outbreaks due to the ingestion of toxic rabbit carrion were recorded by Seddon (1925) and by Rose and Edgar (1930).

In sheep the disease occurs during the dry summer period, from November to May, throughout the wheat-belt and Great Southern portions of Western Australia. It disappears rapidly with the advent of winter rains and green feed. Sheep of all breeds and at all ages appear to be susceptible, though the heaviest losses occur in pregnant and lambing ewes. A clinical description of the disease and the post-mortem appearances are given by Bennetts (1933). An account of the occurrence of the disease with a description of some of the present experiments and their application to farming practice is also given by Underwood and Shier (1935).

The principal cause of the disease is the ingestion of rabbit carrion infected with *Cl. botulinum*. Bennetts (1933) has cultivated *Cl. botulinum* (Type C) from such carrion. Rabbits are present in large and increasing numbers in the affected areas. During the long summer period on poor, dry grazing, the sheep develop an acute depravity of appetite expressed by a craving for rabbit carcases and other carrion. This craving has been commonly attributed to phosphorus deficiency, though very variable results have attended the use of phosphatic licks. Some farmers claim good results in controlling the craving, while others assert that no such benefit has accrued from the licks, even when consumed in large amounts. A proportion of farmers also claim that the feeding of conserved fodders such as hay, silage, or oats during the summer period reduces the incidence of the disease.

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As the evidence appeared so conflicting and the losses from the disease were increasing in spite of the more general use of phosphatic licks, it was deemed imperative to obtain reliable information as to the cause of the pica and possible means of control.

MECKERING EXPERIMENTS, 1933-4.

The grazing of a farm at Meckering, where severe losses from botulism had previously occurred, was leased, and experiments started in the summer of 1933-4. These were designed to ascertain the effects on sheep of supplementing the dry grazing with a phosphatic lick and other feeding stuffs. Particular interest was attached to the effects on:

(1) the incidence and control of the pica;
(2) the growth and wool yields;
(3) the blood phosphorus levels.

Experimental Procedure.

A flock of 100 merino weaners was weighed and divided into five even groups of twenty. The group treatments were as follows:

Group 1	Control: grazing only.
„ 2	Grazing plus phosphatic lick *ad libitum*.
„ 3	„ „ 1 lb. oats per sheep per day.
„ 4	„ „ 1 lb. linseed cake per sheep per day.
„ 5	„ „ ½ lb. wheaten chaff plus ½ lb. oats per sheep per day plus phosphatic lick *ad libitum*.

The sheep were run together in the day time. Each evening (except Sundays) they were yarded, divided into groups, and given their food supplements. At dawn they were turned out to graze once more. Supplementary feeding commenced on 7/12/33, and continued until 2/5/34. For most of the period the food supplements were fully consumed. At the end of February the ration of linseed cake in Group 4 was changed to a mixture of equal parts of meatmeal and oats at the rate of ½ lb. per sheep per day. Only very small amounts of this were consumed. The phosphatic lick was given in the form of Dicalcic Lick with the following composition:

Dicalcium phosphate	45 parts
Common Salt	40 „
Molasses	5 „
Water	10 „

The lick contained 18 p.c. P_2O_5, and proved very palatable to all sheep having access to it. Throughout the dry period it was consumed regularly at the rate of 15 gm. of the lick (3 gm. P_2O_5) per sheep per day in both groups 2 and 5. As with the other supplements, the consumption of lick fell with the advent of green feed.

Climatic and Grazing Conditions.

The climate at Meckering, which is situated about 80 miles east of Perth, is typical of the areas where botulism occurs. It is of the Mediterranean type with moist winters and long hot dry summers. The monthly rainfall figures are given in Table 1.

TABLE 1.

Monthly Rainfall at Meckering.

Year.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total.
1934	75	21	311	370	153	288	246	99	93	12	107	10	1785
1935	67	44	26	72	86	177	273	211	92	91	0	10	1149
Mean 1898–1934	27	44	76	87	205	288	306	225	133	102	44	30	1567

Young green pasture appears with the first rains, usually in May. The herbage consists most entirely of poor annual species which produce very little bulk until about August or eptember, and which quickly mature and dry off with the first signs of summer in October or November. Normally, no new growth takes place until the following May. The chemical composition follows the seasonal trend. In June the herbage is comparatively low in fibre and high in protein and phosphorus. As the season progresses the fibre rises, and the protein and phosphorus contents fall, until by the following March and April, owing to the long dry period and the selective grazing of sheep and rabbits, the herbage is very scanty and exceedingly poor in quality.

Analyses of the grazing available at Meckering are given in Table 2. The samples in each case are selected to conform as far as possible to the material actually consumed by the sheep, a factor of extreme importance with such selective grazers.

TABLE 2.

Pasture and Stubble Analyses—Meckering, 1933-4.

Means of Two Samples. Percentages on Dry Matter.

Period.		Crude Protein.	P_2O_5.	CaO.
December, 1933	Mature pasture	7·43	0·25	1·08
December, 1933	Wheat stubble	6·75	0·32	1·26
March, 1934	Stubble, weathered and grazed	3·91	0·12	0·76
June, 1934	Young and green pasture	17·89	0·64	1·69
August, 1934	Green and fully grown pasture	13·11	0·70	1·42
October, 1934	Maturing pasture	10·55	0·48	1·91

During December the experimental sheep grazed on dry pasture of the type mentioned in the previous paragraph, but for most of the rest of the summer they were run on wheaten stubble, as is usual in these areas. The feeding value of such stubble lies almost entirely in the missed grain and ears, and dry bottom growth of grasses and clovers which become eaten out as the summer advances. Abnormally early rains fell in March, 1934, producing a small but increasing amount of green feed from that time onward.

The Incidence and Control of the Pica, 1934.

The incidence of pica was tested by exposing dried rabbit carcases at frequent intervals to the different groups in small yards, and noting which sheep ate the carcases. In this way a record was obtained of the relative incidence of the craving among the various groups. The carcases were those of rabbits, trapped, gutted, and dried out of contact with the soil to minimize the risk of their containing botulinus toxin.

During December and January the tests were entirely negative, but early in February four of the control group and one member of the lick group (Group 2) were observed eating the carcases. A fortnight later five of the control group and

two of the lick group exhibited pica, but still none of the sheep receiving supplements of oats, linseed, or chaff and oats showed any interest in the carcases.

At this time a number of tests were made with putrid and with moist fresh carcases, as well as with fresh bones and old bleached bones. In all cases a complete absence of craving for these materials was observed.

With the advent of green feed, following on the very heavy rains at the beginning of March, there was a complete disappearance of all signs of pica in the experimental sheep within two weeks.

No losses from botulism were sustained in the experimental flock in 1934, due, no doubt, to the abnormally early rains and short dry period. The losses throughout the State generally were also very small.

The Influence of the Supplementary Feeding on Growth, 1934.

The sheep were weighed at approximately monthly intervals throughout the experiment. Weighings were continued throughout the subsequent green feed period, after supplementary feeding had finished, to see how far any advantage accruing from the supplements would be carried forward into the subsequent life of the sheep.

The average live weights of the groups are given in Table 3.

TABLE 3.

Live Weights of Sheep, 1934.

Average of Groups of 20 Sheep in lb.

Group.	7/12/33	4/1/34	7/2/34	6/3/34	10/4/34	4/5/34	1/6/34	16/7/34	28/8/34	17/10/34*	3/12/34
1. Control	50	47	47	48	48	55	59	82	83	92	99
2. Phosphatic lick, *ad lib.*	50	48	47	46	46	54	58	81	85	93	99
3. 1 lb. oats per sheep per day	50	53	56	59	60	67	70	90	90	97	103
4. 1 lb. linseed cake per sheep per day	50	56	60	62	62	70	72	91	92	96	100
5. ¾ lb. oats and ¼ lb. chaff, plus lick	50	50	51	53	53	61	64	85	85	94	100

Period of supplementary feeding. Green grazing only.

* Shorn 5/10/34.

The weights indicate that the dry grazing available to the controls was not only insufficient to promote growth in these young sheep, but did not even provide a maintenance ration during the first four months of the experiment. The group consuming the phosphatic lick showed a similar small fall in weight, and it was evident that phosphorus deficiency alone was not limiting growth at this time. On the other hand, the sheep receiving the heavy supplements of oats and of linseed cake grew at almost optimum rates, and at the end of April were 12-14 lb. heavier than the controls. The group receiving the small supplement of chaff and oats grew slightly during this period.

Following the dry period all groups rapidly put on weight, and by the end of November, two months after shearing, the sheep of the control and the lick groups had virtually overtaken those of the fed groups. It is evident that the period on the low plane of nutrition did not reduce the ultimate size attained by the sheep compared with those on a high plane of nutrition throughout. This bears out the conclusion of Hammond (1932) that "circumstances causing retardation of growth temporarily are followed by periods of accelerated growth", but this particular case lends no support to his claim that "nutritional conditions in early life limiting growth can materially affect the ultimate development of the animal". It is probable, however, that the conditions limiting growth in the present experiments did not come early enough in the life of the sheep, nor were they severe enough, to produce the effect noted by Hammond.

The Levels of Inorganic Phosphorus in the Blood, 1934.

It has been shown by DuToit, Malan, and Rossouw (1930), and Martin and Peirce (1934), that the concentration of inorganic phosphorus in the blood of sheep (as with cattle) is a reflection of the amount of phosphorus in the diet, and that the determination of the level of inorganic phosphorus in the blood represents a quick and reliable means of diagnosing phosphorus deficiency or sufficiency. Accordingly, it was decided to carry out a series of blood phosphorus tests on a number of the experimental sheep, with the object of ascertaining

(1) the normal seasonal values occurring under typical wheat belt conditions;
(2) the effect of a phosphatic lick and of food supplements on these values during the dry summer period.

It is extremely difficult, as pointed out by Martin and Peirce (1934), to state what is the normal amount of inorganic phosphorus in the blood of sheep, owing to the large variation which exists not only in different individuals on the same diet, but in the same individual from time to time. Nevertheless, it is abundantly clear that, although the upper limit is determined by the individuality of the animal (Watson, 1933b), the lower limit is normally determined by the intake of phosphorus from the food. Levels of 4-4·5 mg. P per 100 c.c. blood or over may be taken as indicating an adequate intake from the diet, while levels consistently below this among a number of sheep would suggest phosphorus deficiency. Under conditions of starvation, however, it is evident from the work of Watson (1933a) that much higher levels might be obtained.

Five typical sheep from each of groups 1, 2, 4, and 5 were bled at approximately monthly intervals throughout 1934. Blood was taken from the jugular vein, and 5 c.c. transferred immediately into 20 c.c. of 10 per cent. trichloracetic acid. After shaking, the mixture was filtered and the inorganic phosphorus determined on the filtrate within two hours by the method of Malan and Van Der Lingen (1931). The results are given in Table 4.

TABLE 4.

Levels of Inorganic Phosphorus in Blood.

Mg. P. per 100 c.c. Blood.

Group 1.	5/1/34	8/2/34	16/3/34	20/4/34	1/6/34	16/7/34	14/9/34	19/10/34	4/12/34
Group 1. Control.									
Sheep No. 2	5·9	4·5	8·0	5·2	7·1	5·2	5·0	4·7	4·9
„ „ 5	6·2	5·2	6·9	4·9	8·9	6·6	5·5	4·5	3·9
„ „ 16	6·9	6·1	6·6	5·0	7·1	7·7	6·1	5·5	5·4
„ „ 18	5·6	4·6	5·2	5·2	7·4	4·9	6·7	4·6	4·4
„ „ 19	6·6	5·6	6·6	4·8	7·7	4·9	5·4	4·9	5·2
Mean	6·2	5·2	6·7	5·0	7·6	5·9	5·7	4·8	4·8
Group 2. Lick fed, 7/12/33 to 2/5/34.									
Sheep No. 23	7·1	6·1	6·6	5·4	8·9	6·7	5·0	4·7	4·6
„ „ 29	5·6	4·6	6·4	4·9	8·9	6·6	7·4	4·7	5·5
„ „ 39	5·2	5·8	5·5	4·7	7·0	5·6	5·5	4·9	4·7
„ „ 48	5·2	5·4	5·8	5·2	7·9	6·0	5·8	4·1	5·0
„ „ 44	5·7	5·2	4·7	4·3	6·7	5·4	6·9	4·9	4·8
Mean	5·8	5·4	5·8	4·9	7·9	6·1	6·1	4·7	4·9
Group 4. Linseed cake and later meatmeal fed 7/12/33 to 2/5/34.									
Sheep No. 64	7·9	7·7	8·1	5·7	7·2	5·3	5·0	4·1	5·2
„ „ 65	7·2	6·1	6·5	4·6	5·5	5·5	4·2	3·0	4·6
„ „ 72	8·9	8·4	6·4	4·3	6·0	5·8	4·4	4·2	4·4
„ „ 73	7·1	6·2	9·6	5·0	9·9	5·7	5·1	4·7	4·5
„ „ 80	7·8	7·5	5·5	4·7	5·4	5·2	5·2	3·8	4·9
Mean	7·8	7·2	7·2	4·9	7·0	5·5	4·8	4·0	4·7
Group 5. Oats, chaff, and lick, 7/12/33 to 2/5/34.									
Sheep No. 86	—	—	6·0	5·2	7·3	8·4	5·7	5·7	5·7
„ „ 89	—	—	6·6	5·0	8·9	6·6	5·7	5·4	5·1
„ „ 92	—	—	5·2	5·5	8·5	4·9	7·0	4·1	5·2
„ „ 6	—	—	7·0	5·5	9·9	5·8	6·1	5·7	4·7
„ „ 67	—	—	4·7	4·4	7·8	4·9	5·3	5·2	3·2
Mean	—	—	5·9	5·1	8·5	6·1	6·0	5·2	4·8

High values were sustained generally by the control sheep throughout the year. The individual fluctuation is large, but no low values suggestive of phosphorus deficiency occurred, even in February, when the dry stubble grazing contained only small concentrations of phosphorus. The levels of the sheep in Group 2, which received the phosphatic lick, are closely similar to those of the controls.

For the first three months of the experiment the linseed-fed sheep (Group 4) showed significantly higher blood phosphorus values than those of the other groups. During the green feed period all groups were very similar. The values in general were higher in June, July, and August than during the earlier and later parts of the year.

MECKERING EXPERIMENTS, 1934-5.

The abnormally short dry period in the 1933-4 season and the consequent very low incidence of pica and of botulism, made it imperative that further experiments be carried out in the summer of 1934-5.

The same farm at Meckering and the same sheep were used for the work. These hoggets of mixed sex were weighed, and divided into seven even groups of twelve. The treatments of the groups were as follows:

Group 1	Control: grazing only (yarded at night).
„ 2	Grazing plus phosphatic lick *ad libitum*.
„ 3	„ „ salt *ad libitum*.
„ 4	„ „ ½ lb. wheat per sheep per day plus phosphatic lick *ad libitum*.
„ 5	„ „ 2 oz. meatmeal per sheep per day.
„ 6	„ „ ½ lb. chaff per sheep per day plus phosphatic lick *ad libitum*.
„ 7	„ only (turned out to graze all night).

As in the previous experiment, the sheep were run together in the daytime, brought in at night (except Sundays) for their supplements, and turned out again at dawn to graze. Feeding commenced on 10/12/34, and continued until 13/6/35.

For almost the whole of the period the rations given were fully consumed, including the meatmeal. The phosphatic lick (Dicalcic lick) again proved extremely palatable, and was taken readily by all sheep having access to it. The average consumption was the very high one of about 30 gm. of the lick (6 gm. P_2O_5) per sheep per day in Group 2, 24 gm. (5 gm. P_2O_5) in Group 4, and about 15 gm. (3 gm. P_2O_5) in Group 6. The mean consumption of salt in Group 3 ranged from 15–25 gm. per sheep per day.

The consumption of lick and salt by these groups fell below these values in May and June, when a certain amount of green feed was available.

Climatic and Grazing Conditions.

From the rainfall records given in Table 1 it is seen that the dry period of the 1934–5 summer was very much longer and more severe than that of the previous year, and rather longer than the average year.

The grazing was similar in composition and amount to that of the previous year for the early part of the summer, but by April and May it had become extremely poor and scanty. It consisted principally of oaten stubble from which the grain and most of the small annual clovers had been eaten. A selected sample of this herbage taken on 8/5/35 had the following composition on a dry matter basis:

Crude protein 2·78 p.c.; P_2O_5 0·15 p.c.

Very small and almost negligible amounts of green picking were also available during May, and larger quantities in June.

The Incidence and Control of the Pica, 1935.

The incidence of the pica was tested as before by exposing dried rabbit carcases to the different groups in small yards.

The tests were entirely negative until the end of January, when three sheep of the control group, five of the salt group, two of the meatmeal, and one of the chaff plus lick group gave evidence of pica. Those sheep receiving lick alone and wheat plus lick, as well as those turned out to graze at night (Group 7) were so far free from craving.

During February and March a more general pica developed in all groups except that receiving the supplement of wheat plus lick (Group 4). By the middle of April, with the continuance of the dry weather, practically the whole of the sheep in the control, the lick, and the salt groups, and nearly half the sheep in the

meatmeal and chaff-fed groups had developed pica. With the exception of one animal the members of the wheat group remained free from craving throughout the summer.

Following on a light fall of rain a very small amount of green "picking" became available during May, and the pica became slightly less pronounced in all affected groups. With the definite advent of green feed in June all signs of it rapidly disappeared.

During the period when the craving for the dried carcases was most intense, tests were made with fresh and with putrid carcases, and also with fresh bones and old bleached bones. As in the previous year none of the experimental sheep touched these materials. Tests at this time with pieces of clean dried rabbit meat, however, gave positive results in many cases, the distribution throughout the groups being closely similar to that obtained with the dried carcases. When eating the carcase it was noticed that the sheep carefully picked the meat from the bones. It is evident that the pica is not an osteophagia, and that it can best be described as a sarcophagia.

An outstanding characteristic of sheep in many parts of Western Australia during the summer period is their liking for dry rabbit faeces. Although such faeces were considered by Bennetts (1933) to be very unlikely sources of the toxin, a trial with the experimental sheep was thought to be of some nutritional interest. In tests carried out at the end of February practically every sheep in each group displayed a marked liking for the rabbit faeces. The possibility that the craving was in some way associated with the chemical composition of the faeces could not be overlooked. Accordingly, four samples were collected and the analyses obtained. They were found to be extremely even in composition, though heavily contaminated with soil. The mean composition is given in Table 5.

TABLE 5.

Mean Composition Rabbit Faeces (four samples).

Percentages Air-Dry Sample.

Moisture.	Ether Extract.	Crude Fibre.	Crude Protein.	Total Ash.	Soluble Ash.	P_2O_5.	Chlorine.
9·73	1·72	30·61	7·52	15·10	3·65	0·51	0·71

Analyst: B. L. Southern, A.A.C.I.

The analyses given reveal nothing which would suggest a reason for the liking shown by sheep for the faeces.

Losses from Botulism, 1935.

The following losses from botulism were sustained:

Group 1, two; Group 2, nil; Group 3, four; Group 4, two; Group 5, one; Group 6, one; Group 7, two.

It is strongly suspected that some at least of these losses were due to carrion-infected water-supply (soaks), a source of toxin which would be equally available to all sheep independently of whether they exhibited pica or not. For this reason, and because of the small groups involved, it is felt that the actual losses in the different groups bear little relation to the effectiveness of the various treatments in controlling pica. For instance, there were no deaths in the lick-fed group (Group 2), although almost every animal in the group had developed a craving for carcases by the middle of April.

The Influence of the Feeding on Live Weight, 1935.

The weighings throughout 1935 are given in Table 6.

TABLE 6.

Live Weights of Sheep, 1935.

Averages of Groups of Twelve Sheep in lb.

Group.		3/12/34	8/1/35	11/2/35	12/3/35	28/3/35	11/4/35	9/5/35	23/5/35	14/6/35	6/7/35	13/8/35	25/9/35
Group 1	Control	100	96	88	87	84	77	74	75	68	70	68	86
Group 2	Phosphatic lick *ad libitum*	100	97	90	91	86	79	76	76	69	70	69	86
Group 3	Salt *ad libitum*	100	95	86	87	85	78	77	77	68	70	68	86
Group 4	½ lb. wheat per sheep per day plus lick	100	99	97	99	95	90	89	90	84	82	79	93
Group 5	Meatmeal 2 oz. per sheep per day	100	96	95	96	92	87	85	84	77	78	75	91
Group 6	½ lb. chaff per sheep per day plus lick	100	95	91	92	87	82	80	81	74	73	71	87
Group 7	Grazing control	100	98	89	90	84	78	73	75	67	70	68	82
		Period of supplementary feeding.									Green grazing.		

It is evident from the mean group weights that most of the sheep were full-grown and in fair condition at the commencement of the experiment. The control sheep steadily lost weight for the following six months, the mean weight being about 30 lb. less in June than in the preceding December. It is evident that these sheep were on a large negative energy balance during this period, and that the dry grazing was deficient either quantitatively, qualitatively, or both. That the deficiency is not primarily one of phosphorus is shown by Group 2, whose mean live weights followed closely those of the controls. This amply bears out the findings of the previous year, in which no increased growth was obtained in the lick-fed sheep.

For the first three months of the experiment the ration of ½ lb. wheat per sheep per day plus lick prevented any appreciable loss in weight, but during the rest of the dry period even this supplement was insufficient to maintain a positive energy balance, and by June the group averaged sixteen pounds less than in December. The sheep receiving the supplements of meatmeal and of chaff lost weight steadily, though not to the same extent as the controls and lick-fed sheep.

TABLE 7.

Levels of Inorganic Blood Phosphorus, Meckering 1935.

Mg. P per 100 c.c. Blood.

Group		4/12/34	16/1/35	11/2/35	12/3/35	28/3/35	9/4/35	9/5/35	23/5/35	14/6/35	3/7/35	6/8/35	4/9/35	2/10/35
1	Mean	4·7	4·7	5·0	5·0	5·4	4·8	5·2	5·2	5·1	5·2	5·9	5·0	5·0
Control	Range	3·4–6·6	3·3–6·2	3·9–6·6	3·7–6·2	4·3–7·0	3·8–5·6	4·4–6·4	4·2–5·7	4·2–6·4	3·7–7·0	4·3–7·8	3·9–6·6	4·3–5·5
2	Mean	5·1	4·4	5·5	4·5	5·9	5·2	5·9	6·4	5·9	4·9	5·4	4·6	4·8
Phosphatic lick *ad lib.*	Range	4·4–5·9	3·5–6·3	4·0–7·0	3·9–5·2	4·0–8·2	3·8–6·8	4·6–7·6	5·5–7·3	4·2–8·8	4·3–5·5	4·3–6·6	3·2–5·5	3·7–6·5
5	Mean	4·7	4·8	4·6	5·1	5·2	4·8	5·5	5·8	4·6	5·1	4·7	4·8	5·1
Meatmeal	Range	3·2–5·4	3·6–5·7	3·5–6·6	3·9–6·3	4·2–6·7	4·0–6·4	4·2–7·6	4·9–7·5	3·6–5·2	4·2–6·1	3·3–6·0	4·0–5·7	3·8–6·3
		Period of supplementary feeding.									Period of green pasture.			

For the first two months of the green feed period, little increase in live-weight was shown by any of the groups. During September, however, all groups rapidly increased in weight. As in the previous year the control sheep grew faster, and began to overtake in weight those sheep which had received the food supplements throughout the summer months.

The Levels of Inorganic Phosphorus in the Blood, 1935.

Owing to the large individual variations in the previous year, it was decided to carry out blood phosphorus determinations on the whole twelve sheep of three of the groups in the 1934-5 experiments, and to continue these determinations as in 1933-4 into the green feed period, after supplementary feeding had finished. The same method and bleeding technique was used as before. The sheep tested were those of the control, the lick-fed, and the meatmeal-fed groups. The results are given in Table 7.

The average levels of inorganic phosphorus in the blood of the control sheep showed little variation throughout the year, though the individual variation was again very large. The mean levels did not fall to low values, even during March and April, when the grazing conditions were very poor indeed, and a selected sample of the herbage was found to contain only 0·15 p.c. P_2O_5 in the dry matter.

As in the previous summer the consumption of the phosphatic lick had no consistent effect in raising the blood phosphorus levels, though on occasions when bled very shortly after the lick had been eaten, high values were obtained. The levels in the meatmeal-fed sheep were very similar throughout to those of the control group. It must be noted that the same large variation existed within the lick and the meatmeal groups as within the control group. The occasional low values obtained in odd sheep are apparently individual fluctuations produced by factors other than that of the phosphorus intake in the diet.

EXPERIMENTS AT BEVERLEY.

During the summers of 1933-4 and 1934-5 experiments of a similar general nature to those at Meckering were carried out at the Avondale State Farm, Beverley, situated about 50 miles south of Meckering, where no losses from botulism had been known to occur. Beverley is characterized by the same long, hot, dry summer conditions as Meckering, but the soil and grazing conditions are in general slightly better. Mated ewes were used for the first year's experiments, and wether weaners for the second.

During the abnormally short summer of 1933-4 no pica could be demonstrated in any of the experimental sheep. Even in the long 1934-5 summer, when the craving at Meckering became acute, comparatively little interest in the rabbit carcases was shown at Beverley. Three of the control sheep on the dry grazing only, and one of the phosphatic lick-fed sheep from groups of ten, exhibited a mild pica.

No interest at all was shown by the sheep receiving supplementary rations of wheat, of meatmeal, and of silage. There were no losses from the disease. The reason for the absence of pica, as evidenced by the carrion tests, is not at present clear.

The live weight figures showed a similar trend to those at Meckering. The mature sheep lost considerable weight during the summer, and the young sheep failed to grow. The phosphatic lick had no effect either in promoting growth in the young sheep or in preventing weight loss in the old. As at Meckering, however, the supplementary feeding gave some help in this direction.

The blood phosphorus figures of the Beverley sheep showed no significant falls from normal values in the summer months in either year. In fact the levels were closely similar to those at Meckering. It is apparent that, under the conditions at Beverley, phosphorus deficiency is not the limiting factor in nutrition, and that the general nutritional findings bear out those obtained at Meckering.

A point of interest arising from the 1933-4 experiments at Beverley with mated ewes is the very marked falls in the inorganic phosphorus levels of the blood, which occurred with most of the ewes a day or two before parturition. In some of the ewes the low values continued for several months before returning to normal levels, while in others the values rose rapidly within a matter of days. As the ewes were on green grazing at the time, containing 0·7-1·0 p.c. P_2O_5 on the dry matter, such a marked effect was rather unexpected, and the matter is receiving further attention during the present season.

DISCUSSION.

It is evident from the large falls in live weight of the sheep during the dry summer months that they are on a very low plane of nutrition at this time. Heavy supplementary feeding is necessary to prevent these losses. Towards the end of the summer the grazing is not only dry and scanty, but exceedingly low in protein and phosphorus. Selected samples of the herbage at Meckering were found to contain only 3-5 p.c. protein and 0·12-0·15 p.c. P_2O_5 in the dry matter. Under these conditions a deficiency of available energy, of protein, and of phosphorus would certainly be expected. The suggestion of available energy and protein deficiency is supported by the experiments, but it is evident that phosphorus deficiency is not the limiting factor in nutrition, since no increase in live weight is brought about by even a large intake of a soluble phosphate lick. Furthermore, the levels of inorganic phosphorus in the blood of the sheep showed no significant falls to low values, even during the late summer period, but remained at a very constant general level throughout the year.

Considering the very low concentrations of phosphorus found in the pasture samples, these results are difficult to understand, unless the phosphorus requirements of sheep are much lower than has previously been supposed; or unless the other nutritive deficiencies of the grazing have the effect of reducing the amount

of dietary phosphorus required. These hypotheses have also been recently put forward by Marston (1934, 1935), who, from a consideration of the more massive skeletal system of cattle and their lower food consumption per unit gain, suggests that growing sheep only require about one-third of the concentration of phosphorus in their diet of that needed by young cattle. He also points out that the analyses of the dry herbage may not truly represent the material actually consumed by the sheep; that their intake of phosphorus might be much greater than the analyses imply, and stressed the marked powers of selective grazing which sheep display. The present authors feel that this criticism is one which might be levelled at many published pasture analyses. In the present investigations, however, every attempt has been made to select for analysis the material actually consumed by the sheep. The effect on composition was sometimes quite marked. For instance, a selected sample of stubble-grazing was found to contain early in the summer 6·8 p.c. protein and 0·3 p.c. P_2O_5, while a sample consisting principally of straw, which made up most of the bulk of the grazing (but was not eaten), contained only 2·3 p.c. protein and 0·07 p.c. P_2O_5.

The suggestion that the dietary phosphorus requirements of the sheep might be lowered during the summer months by the low level of other dietary constituents, such as available protein and energy, is one that cannot be overlooked, especially in view of the large falls in live weight which occur at that time. It might be suggested, for instance, that the phosphorus set free by tissue destruction (endogenous phosphorus) would be sufficient to reduce appreciably their dietary requirements. Using Marston's (1935) figure of 0·15 p.c. for the phosphorus content of the whole flesh of sheep, calculation shows that even in the control sheep in the 1934-5 experiments at Meckering, which lost an average of 30 lb. live weight in six months, the total amount of phosphorus set free was only about 0·1 gm. P daily. This amount would hardly be sufficient to affect seriously their exogenous requirements. The data so far obtained on the composition of the bones of sheep at the end of the summer period suggest further that these phosphorus reserves have not been drawn upon to an appreciable extent. An attempt is being made in the present summer to induce phosphorus deficiency in a group of young sheep by giving them a supplement to correct the deficiencies of the dry grazing in protein and available energy, without materially increasing their phosphorus intake.

The relation of the nutritional conditions during the summer months to the incidence of the pica is not at present clear. Phosphorus deficiency as the cause of the craving must be ruled out, since a large intake of a soluble phosphatic lick did not prevent its appearance. It is significant also that the sheep displayed no interest in bones, but exhibited a special liking for the fleshy portions of the rabbit carcases. Skin and viscera, however, are also eaten on occasions. The marked taste for rabbit faeces has also been noted. The craving for flesh (sarcophagia) suggests a protein deficiency, if the analogy with osteophagia be allowed, but no

less than half the sheep which were fed with meatmeal displayed sarcophagia. It is possible, however, that these sheep had developed a taste for the dried flesh through receiving the ration of meatmeal, and further trials are being carried out in the present season.

The pica disappears with such remarkable rapidity upon the advent of the first green feed, even when available only in very small amounts, that it seems rather unlikely that it can be caused by a direct deficiency of an essential nutrient or nutrients. It seems more likely that the craving is due to some food error, such as a disturbance of the acid-alkali balance or a physiological upset due to the dry fibrous nature of the food, which the small ration of green succulent pasture is able to rectify. A thorough investigation of this phase of the problem is being made at present, together with an attempt to place the pica upon a more definite physiological basis by a study of the body fluids of sheep while exhibiting pica and after its disappearance.

SUMMARY AND CONCLUSIONS.

A brief account of the occurrence of botulism in sheep in Western Australia is given, together with a description of experiments carried out in the affected areas during the summers of 1933-4 and 1934-5. The experiments were designed to throw light on the effects of various supplementary feeding treatments on

(i) the incidence and control of the pica which impels sheep to consume rabbit carcases, the principal source of the botulinus toxin;
(ii) the growth and live weights; and
(iii) the blood inorganic phosphorus levels of the sheep.

The pastures and stubbles were also examined. By the late summer period they were found to become very poor, dry, and scanty. Samples, selected to conform as far as possible to the material actually consumed by the sheep at this time, were found to contain only 3-5 p.c. protein and 0·12-0·15 p.c. P_2O_5 on the dry matter.

The following results were obtained from the experiments:

(1) Considerable falls in live weight were shown by both young and old sheep on the poor, dry grazing. Heavy supplementary feeding is necessary to prevent these losses. A large consumption of a soluble phosphatic lick had no effect on the live-weight of young or old sheep.

(2) The average levels of inorganic phosphorus in the blood of sheep remained between 4·5-6·0 mg. P per 100 c.c. throughout 1934 and 1935. No falls to low values were evident, even during the late summer period when the phosphorus content of the grazing was very low. No consistent effects on the blood phosphorus levels were produced either by the phosphatic lick or the supplementary foods consumed.

(3) Little evidence of pica was shown in the summer of 1933-4, owing to the abnormally early appearance of green feed, but in the following summer, which was long and severe, the whole of the sheep on the unsupplemented dry grazing exhibited pica, evidenced by a liking for rabbit carcases. The pica appeared to be a sarcophagia, no sign of osteophagia being found. The phosphatic lick consumed had no effect in preventing the pica, though a partial control was given by some of the food supplements.

(4) It is concluded from these findings that this form of pica is not due to phosphorus deficiency, and that the phosphorus requirements of sheep are probably much lower than has previously been supposed. It is suggested further that the pica is caused, not by a direct deficiency of an essential nutrient, but by some food error such as a disturbance of the acid-alkali balance or a physiological upset due to the dry fibrous nature of the diet.

A thorough investigation of this phase of the problem is at present being made, together with an attempt to place the pica upon a definite physiological basis.

REFERENCES.

Bennetts, H. W. (1933): J. Commonwealth C.S. & I. R., 6, p. 137.
DuToit, Malan & Rossouw (1930): 16th Rept. Dir. Vet. Services and An. Ind. U. of S. Afr., p. 313.
Hammond, J. (1932): Growth and Development of Mutton Qualities in the Sheep.
Malan and Vander Lingen (1931): 17th Rept. Dir. Vet. Services and An. Ind. U. of S. Af., p. 443.
Marston, H. R. (1934): Commonwealth C.S.I.R. Bull. 85.
Marston, H. R. (1935): J. Commonwealth C.S. and I.R., 8, p. 293.
Martin and Peirce (1934): Commonwealth C.S.I.R. Bull. 77.
Rose and Edgar (1930): Austral. Vet. J., 6, p. 123.
Seddon, H. R. (1935): N.S.W. Dept. Agric. Vet. Res. Rept., No. 1, p. 7.
Underwood and Shier (1935): J. Dept. Agric., W.A., 12, p. 277
Watson (1933, a): Austral. J. exp. Biol., 11, p. 67.
Watson (1933, b): Austral. J. exp. Biol., 11, p. 197.

PRELIMINARY NOTE ON THE DISSOCIATION OF STAPHYLOCOCCI INTO ALPHA AND BETA TOXIGENIC VARIANTS

by

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(Submitted for publication 10th January, 1936.)

During an investigation of the toxins of 43 strains of *Staphylococcus aureus*, six were encountered which showed an interesting dissociation of their toxigenic properties.[1] These six strains when plated from stock culture on sheep blood agar produced after incubation at 37° C. only colonies surrounded by a clear zone of haemolysis. This zone of complete haemolysis has been shown by previous work (Bryce and Rountree, 1936) to be due to the action of the classical α toxin, and the phenomenon may be termed α haemolysis. However, on suitable subculture, e.g. growth in broth in an atmosphere of CO_2–O_2, or prolonged incubation in broth containing 1 p.c. potassium oxalate, these strains gave rise to 1–2 p.c. of variants, which on sheep blood agar gave the appearance characteristic of β toxin-producing staphylococci, i.e. a wide zone of partial haemolysis which, after refrigeration overnight, is completely haemolyzed ("hot-cold" lysis). With some colonies there was an additional narrow inner zone of α type haemolysis. The nature of the toxins produced by these variants in broth culture as determined by their relative activities in haemolyzing rabbit and sheep cells, and by the use of pure α and β antitoxic sera, corresponded exactly with what would be deduced from the type of haemolysis presented on sheep blood agar.

This variation is of the phasic type, the α and β phases under certain conditions existing together in broth cultures, with the α phase as the predominant type. The α phase is not capable of stabilization, but always tends to produce about 1 p.c. of β variants. This was demonstrated with one strain for six successive subcultures picked on each subculture from single colonies, and for five subcultures with another strain.

The β variants when first produced tend to revert to the α phase. About 75 p.c. of cultures obtained from single colonies on sheep blood agar plates produced on

[1] The technique employed for the growth of cultures on sheep blood agar, and for preparation and titration of toxins, was similar to that described in the previous paper (Bryce and Rountree, 1936).

subculture an equilibrium mixture containing approximately 90 p.c. of α organisms and 10 p.c. of β. The other 25 p.c. of β colonies could be readily stabilized on subculture in CO_2–O_2, and when this had occurred it was not found possible to induce reversion to the α phase. The occurrence of this dissociation could be repeated with fresh subcultures from the stock cultures of the strains.

Sheep blood agar plate after incubation at 37°C. overnight, inoculated from a single β colony and showing both α and β colonies. The α colonies are surrounded by a zone of complete clearing, the zones around the β colonies are only partially haemolyzed.

The instability of these strains, which is manifested in the production of β variants, is probably an inherent characteristic. That it is not due to prolonged culture under laboratory conditions is shown by one of the strains which, on plating direct from the lesion, produced only α haemolytic colonies. Several of these colonies were grown in broth in CO_2–O_2, and immediately produced β variants.

This dissociation appears to be analogous in some respects to that described by Andrewes (1922) as occurring in agglutinogens of the Salmonella group.

The two toxins have previously been shown to be antigenically distinct [Glenny and Stevens (1935), Bryce and Rountree (1936)]; the β toxin is of much

lower toxigenicity for laboratory animals than is the α toxin, and is more restricted in its haemolytic powers for the red cells of various species of animals. This is the first time, so far as I am aware, that dissociation has been reported involving the appearance of variants producing two antigenically distinct toxins. Further work will be done on this subject.

REFERENCES.

Andrews, F. W. (1922) : J. Path. Bact., Lond., 25, p. 505.
Andrewes, F. W. (1925) : J. Path. Bact., Lond., 83, p. 345.
Bryce, L. M. and Rountree, P. M. (in press).
Glenny, A. T. and Stevens, M. (1935) : J. Path. Bact., Lond., 40, p. 201.

THE DETERMINATION OF HAEMOGLOBIN AS GLOBIN PICRATE

by

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(Submitted for publication 24th February, 1936.)

In 1894 Hüfner estimated the carbon monoxide combining power of ox haemoglobin. In this determination the author found it necessary to introduce a number of corrections which had but slender experimental justification, and he finally decided that one gram of dry pure haemoglobin combined with 1·34 c.c. of carbon monoxide. The hypothetical value of Hüfner is still in use to-day in the estimation of the haemoglobin content of human blood from gasometrically determined O_2 and CO combining capacities. The more accurate colorimetric methods for the determination of haemoglobin are based on standards obtained by gasometric oxygen determinations, where the volume of oxygen found in 100 c.c. of blood is translated into grams of haemoglobin by dividing the volume of the oxygen obtained by 1·34, i.e. Hüfner's figure.

Recently, Peters and Van Slyke (1932) pointed out that probably the simplest way to determine exactly the maximum oxygen combining power of 1 gm. of haemoglobin would be the precise determination of the iron content of purified human haemoglobin. This problem, however, has not yet been solved, because the preparation of pure haemoglobin is a very difficult one.

During a consideration of this problem, however, it was thought that the question could probably be attacked in a different way. Instead of trying to ascertain the iron content of 1 gram of pure human haemoglobin (in a molecule of which it amounts only to about 0·34 p.c.), an attempt was made to measure, in some form or other, the globin content of the haemoglobin, since this comprises 96·3 p.c. of the molecule. For this purpose the acid-combining power of globin was examined. Globin readily combines with acids. It has been known for some time that it forms a hydrochloride and a phosphate of fairly constant composition (Ishiyama, 1928). The picric acid content of globin picrate was found to be 25·7 p.c., with a maximum deviation of 0·4 p.c., or, in other words, 1 gm. of globin combines with 0·33 gm. of picric acid. The determination of the picric acid content of the globin picrate was executed by titration with methylene blue, a method

which is accurate and simple because the methylene blue does not react appreciably with the globin, but only with the picric acid (Bolliger and Goulston, 1935).

By adding alcoholic picric acid to red cells, a similar globin picrate was obtained, the chromogen, namely haematin, remaining in solution. It was therefore thought that this reaction, the picric acid-combining power of globin, could be applied to the determination of globin or haemoglobin. Consequently haemoglobin solutions of approximately known haemoglobin content were added to alcoholic picric acid, and the precipitate obtained was washed with alcohol and ether. The picric acid content of the precipitate was then determined by titration with methylene blue. The results obtained indicated that the amount of globin picrate formed corresponds within about 2 p.c. to the haemoglobin present as determined by the acid haematin method, assuming the correctness of the current view that haemoglobin consists of one molecule of globin and one molecule of haematin. These experiments established the possibility of determining haemoglobin as globin picrate. The procedure, however, is somewhat lengthy. The globin has first to be precipitated from a haemoglobin solution free from serum proteins, white cells, and stroma. After this the precipitate, following its isolation, has to be washed free from any excess of picric acid present. Finally the precipitate has to be dissolved in alkali and neutralized before the titration can be executed. The possibility was therefore examined as to whether the globin content of a haemoglobin solution could be determined without actually isolating the precipitate of globin picrate, by titrating the excess of picric acid added to the haemoglobin solution.

In this series of determinations the haemoglobin content of the solution tested was determined by measuring the oxygen-combining power, using the gasometric methods of Van Slyke and Haldane.

The procedure adopted for the determination of haemoglobin as globin picrate is as follows: Into a 25 c.c. volumetric flask containing 5 c.c. of 0·1N alcoholic picric acid, is placed 1 c.c. of 8–16 p.c. haemoglobin solution. To avoid the formation of lumps the solution has to be added slowly, directly into the picric acid and with stirring. The mixture is then brought to boiling point on a water bath, and kept there for about 10 minutes. This step ensures that the reaction takes place throughout the whole of the precipitate, because the freshly-formed globin picrate is to a large extent soluble in boiling alcoholic picric acid. The mixture is then allowed to stand at room temperature for at least an hour, during which time the dissolved globin picrate comes out of the solution again. The mixture is then made up to the 25 c.c. with alcohol, mixed and filtered through a fast filter, after which the amount of picric acid present in an aliquot part of the filtrate is determined by titration with methylene blue. The amount of globin present is calculated by subtracting the amount of picric acid recovered, expressed in grams, from the amount of picric acid originally added to the haemoglobin solution. The result is then multiplied by a factor which converts the picric acid used into globin. In

experiments (Bolliger and Goulston, 1935) it was found that globin picrate contains on the average 25·7 p.c. picric acid. In this case the factor which converts the picric acid used into globin is 2·89. The amount of globin so found is then converted into haemoglobin by multiplying with 1·036. As shown in the table the results obtained under these conditions agree remarkably well with the results

Exp. No.	Haldane gm. haemoglobin per cent.	Van Slyke gm. haemoglobin per cent.	Globin Picrate gm. haemoglobin per cent.	
1.	12·6	12·4	12·37	(haemoglobin solution)
2.	13·1	13·4	13·39	" "
3.	9·9	10·0	10·10	" "
4.	—	10·6	10·75	" "
5.	10·6	—	10·67	" "
6.	15·5	15·6	15·37	" "
7.	12·4	12·5	12·66	(washed cells)

Results obtained by the determination of haemoglobin as globin picrate compared with the gasometric methods of Haldane and Van Slyke.

obtained by the gasometric methods of Haldane and Van Slyke. Furthermore, this determination can also be applied to washed red cells, which still contain the normally occurring number of leucocytes. In this case the result will be slightly too high (Experiment 7).

SUMMARY.

The determination of haemoglobin can be carried out by virtue of the acid-combining power of globin. This has been demonstrated in the case of its combination with picric acid. The results obtained agree well with those obtained by the standard gasometric determinations, and lend further support to Hüfner's conversion factor established in 1894.

ACKNOWLEDGMENTS.

I wish to thank Dr. A. J. Canny for his interest, and for the carrying out of the oxygen determinations by the method of Haldane. My thanks are also due to Miss Dorothy Dark for her valuable technical assistance.

REFERENCES.

Bolliger, A. and Goulston, E. (1935): Austral. J. exp. Biol., 13, p. 23.
Hüfner, G. (1894): Arch. Anat. Physiol., p. 130.
Ishiyama, N. (1928): Z. physiol. Chem., 176, p. 294.
Peters, J. P. and Van Slyke, D. D. (1932): Quantitative Clinical Chemistry, London, 1, p. 524.

THE RELATIONSHIP OF THE COMPLEMENT FIXATION OPTIMUM TO THE AGGLUTINATION OPTIMUM

by

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(Submitted for publication 27th April, 1936.)

Whilst studying the fixation of complement by the potato virus "X" in the presence of its homologous antiserum, Spooner and Bawden (1935) observed the occurrence of zones of optimal fixation when a range of dilutions of purified antigen was used against a constant dilution of antiserum. With any particular dilution of antiserum there was a dilution of antigen which gave a greater degree of fixation than any other antigen dilution. The ratio of antigen to antiserum in those mixtures giving optimal fixation did not remain constant, and with higher dilutions of the antiserum the zone of optimal complement fixation moved towards the side of antigen excess.

As no explanation of this phenomenon was apparent, it was suggested by Professor H. R. Dean that this point be investigated further, and with this object the experiments here described were carried out.

EXPERIMENTAL WORK.

Materials. A smooth strain of Type III pneumococcus was used as antigen. This strain was seeded into a number of 80 c.c. tubes of Wright's (1933) broth, and after incubation for 24 hours the organisms were recovered by centrifugation, and after washing once with saline were resuspended in saline and stored as a thick suspension of approximately 20×10^9 organisms per c.c., to be diluted as required.

The homologous antiserum had been prepared in the rabbit in the usual way.

As a source of complement the fresh serum of a guinea-pig was used.

The haemolytic system consisted of sheep's red cells and antisheep rabbit serum.

Experiment 1. Determination of Optimal Agglutination Ratio of the Antigen to Antiserum. A technique similar to that described by Miles (1933) was used. To a range of dilutions of antigen was added an equal volume of antiserum diluted 1 : 400, and the tubes were incubated in an illuminated water bath at 55° C. so that readings could be made without removing the tubes from the bath. As the result

[1] The main portion of this work was carried out while the author was Gwynaeth Pretty Research Student, Department of Pathology, University of Cambridge.

of several observations, it was determined that a suspension containing 3,000 x 10^6 per c.c. flocculated most rapidly in the presence of a 1 : 400 dilution of antiserum.

Experiment 2. Determination of Optimal Complement Fixation Ratio of Antigen to Antiserum. Ten rows of tubes, six tubes to each row, were set up. To

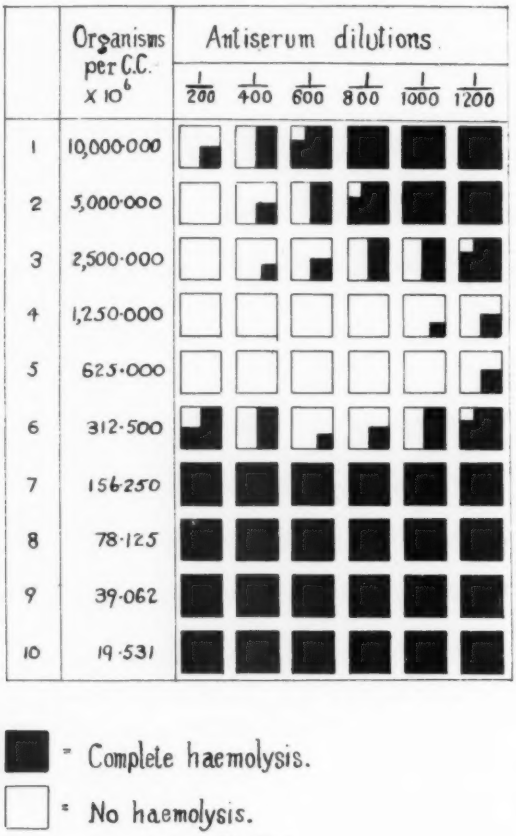

Figure 1.

each tube of the first row was added 0·5 c.c. of a suspension containing 10,000 x 10^6 organisms per c.c. To each tube of the second row was added a similar volume of a suspension containing 5,000 x 10^6 organisms per c.c., and so on, each tube receiving 0·5 c.c. of a bacterial suspension of a strength indicated in the second column of Fig. 1. Complement was then added as 0·5 c.c. of a 1 : 10 dilution of fresh guinea-pig serum, followed by 0·5 c.c. of a dilution of antiserum as indicated in

Fig. 1, the first tube of each row receiving serum diluted 1 : 200, the second tube 1 : 400, and so on. The necessary controls of antigen and complement and serum and complement were included. All the tubes were then placed in the cold-room at 2° C. for two hours. The haemolytic system was then added, and after incubation at 37° C. for one hour readings were made. The results are recorded in Fig. 1.

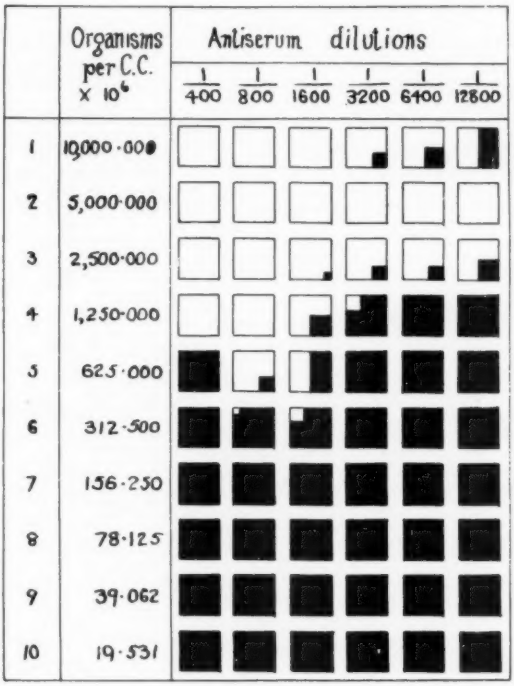

Figure 2.

It will be noted that in the lower dilutions of antiserum there is a wide zone in which complete fixation occurs, but that, with increasing dilutions of the antiserum, this zone narrows considerably. Also, in the lower serum dilutions the mixtures in which the maximal complement fixation occurs lie on the antibody excess side of that in which optimal flocculation takes place, but with increasing serum dilutions the complement fixation optimum approaches nearer to the flocculating optimum, and at a serum dilution of 1 : 1,200 passes to the antigen excess side of it.

An even more clear-cut result was obtained with a strain of *Brucella abortus* and its homologous antiserum. A typical finding is recorded in Fig. 2. With the

antiserum diluted 1 : 400, optimal agglutination occurred with an antigen strength of 5,000 x 10^9 organisms per c.c.

In the lowest dilution of antiserum the centre of the zone of complement fixation falls near the agglutination optimum, but with increasing antiserum dilutions the zone of complement fixation narrows and moves to the antigen excess side of the agglutination optimum.

Tube No	Time at which antiserum added	Length of agglutinating time	Complement Fixation Readings
1	—	105 min.	
2	15 mins	90 "	
3	30 "	75 "	
4	45 "	60 "	
5	60 "	45 "	
6	75 "	30 "	
7	90 "	15 "	
8	105 "	—	
9	—	—	

Figure 3.

In all these experiments complement fixation was allowed to take place at ice-box temperature, with the purpose of preventing any marked alteration of the physical condition of the system. As shown by Dean (1911, a and b; 1912) and Goldsworthy (1928) the physical state of the antigen-antibody complex does determine the amount of complement fixed and the relationship of the zone of fixation to the flocculation optimum.

That the physical condition of the reagents is important when a large particulate antigen is used is demonstrated by the following experiment.

Experiment 3. To a series of nine tubes numbered from 1 to 9 was added 0·5 c.c. of a suspension of pneumococcus type III, and then all were placed in a water bath at a temperature of 34° C. Immediately 0·5 c.c. of a 1 : 400 dilution of homologous antiserum was added to tube No. 1. After 15 minutes a similar volume of the same dilution of antiserum was added to tube No. 2, and so on, so that each tube up to and including No. 8 received a volume of diluted antiserum 15 minutes after the one preceding it. Immediately after adding the serum to tube No. 8, 0·5 c.c. of saline was added to tube No. 9, and all tubes then received 0·5 c.c. of a 1 : 10 dilution of fresh guinea-pig serum. Two hours at ice-chest temperature was allowed for fixation, when the haemolytic system was added, and after incubation at 37° C. for one hour readings were made.

Several such experiments were performed, and the result given in Fig. 3 is typical of all those obtained.

It will be seen that in tubes Nos. 5 to 8 there was complete fixation of complement, but that in Nos. 1 to 4 there has been only partial fixation. In these tubes the antigen and antibody have been in contact for an hour or more, and at the stage when complement was added the suspension has become visibly particulate, ranging from large particles, easily visible to the naked eye in No. 1, to very fine particles just visible with the aid of a lens in No. 4. Alteration in the physical condition of the suspension after addition of the complement was prevented as far as possible by placing the system during fixation in the ice-chest.

DISCUSSION.

In his studies on complement fixation in precipitation, Goldsworthy (1928) showed that the relationship of the complement fixation optimum to the flocculation optimum was not a simple one, and that the size of the "particle" of precipitate formed from the interaction of antigen and antibody was important in determining the amount of complement fixed. It would appear that between particles of the newly-formed antigen-antibody complex and those which are visible there is a particle which, providing a relatively large surface, takes up a maximum of complement. Particles of larger or smaller size fix less complement.

In a precipitating system then, "Complement fixation is a function of the size of the particle of a precipitate" (Goldsworthy, *loc. cit.*).

If this is so, then in a system in which the antigen is particulate *ab initio*, as for example when a suspension of some organism is used as antigen, the process of complement fixation can commence at the moment when antigen, antibody, and complement are brought in contact, and the amount fixed would be governed by the length of time allowed for fixation, the degree of sensitization of the organisms, and the number of sensitized particles upon which complement can be "fixed".

If we standardize the time factor to a period of sufficient length to allow maximum possible fixation, we can study the relationship of the remaining two factors, one to the other.

In a constant antigen-varying antiserum system the zone of complement fixation is usually considerably wider than that in which agglutination occurs, extending far into the antigen excess side of the agglutination optimum. Organisms only very slightly sensitized are capable of fixing several M.H.D. of complement.

With low dilutions of antiserum in a constant antibody-varying antigen system, as described in this paper, the mixture which gives optimal agglutination contains a large number of sensitized organisms upon which complement can be "fixed".

With increasing dilution of the antiserum the optimum moves towards the dilute antigen side, and although each organism in the optimal tube should be sensitized to the same degree as those in any other optimum, the number of sensitized particles is considerably less than in a low antiserum dilution optimum, and consequently the total amount of complement fixed must be less. On the antigen excess side of this optimum there is a zone in which there is a larger number of organisms partially sensitized, and the results of experiments with pneumococci and *Brucella abortus* show that when the number of sensitized organisms in a mixture containing optimal proportions for agglutination is small, the total amount of complement fixed is insignificant, and may be infinitely less than that fixed by a greater number of organisms only partially sensitized.

SUMMARY.

1. Experiments to study the relationship between the complement fixation optimum and the flocculation optimum in mixtures of antiserum and a particulate antigen are described.

2. A large number of organisms partially sensitized may fix more complement than a smaller number optimally sensitized for flocculation.

ACKNOWLEDGMENT.

In conclusion I wish to thank Professor H. R. Dean for continued encouragement and advice during the performance of the earlier portion of this work.

REFERENCES.

Dean, H. R. (1911): Proc. R. Soc. Med., 4 (Path. Sect.), p. 251; 1911, *ibid.*, 5 (Path. Sect.), p. 62.
Dean, H. R. (1912): J. Hyg., Camb., 12, p. 259.
Goldsworthy, N. E. (1928): J. Path. Bact., Lond., 31, part i, p. 220.
Miles, A. A. (1933): Brit. J. exp. Path., 14, p. 43.
Spooner, E. T. C. and Bawden, F. C. (1935): Brit. J. exp. Path., 16, p. 218.
Wright, H. D. (1933): J. Path. Bact., Lond., 37, p. 257.

INVESTIGATIONS INTO THE NATURE OF THE CONDITION KNOWN AS "FLOATING YOLK" IN EGGS

by

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(Submitted for publication 30th April, 1936.)

During the past twelve months an abnormality has appeared in a large percentage of fowls' eggs being examined on the various packing floors in Australia. The condition is characterized by an excessive mobility of the yolk with, in some cases, an increase in the density of the shadow cast by the yolk when the egg is candled. The term "floating yolk" is used to designate this condition, and, as in the greater percentage of affected eggs the yolk rises to its uppermost limit within the egg in whatever way the egg is turned, the term is self-explanatory.

Similar conditions have been reported elsewhere. Halnan and Day (1935), in an examination of large numbers of eggs in West Suffolk County during the years 1931-34, reported the occurrence of several abnormal conditions, including "floating yolk", but the number of eggs showing this fault was very small, and never exceeded 0·260 per cent.

The information available, however, does not permit us to identify, with certainty, the condition described by the English workers with that observed here. In both cases the term "floating yolk" may denote, not an entity, but several conditions producing almost identical results.

Some confusion has been caused by the fact that another abnormality has become prominent recently in certain districts. This is the condition known as "watery white" (see Anderson, 1936). The two conditions are, however, easily distinguished, since in "watery white" the characteristic mobile condition of the yolk is absent, and the eggs appear more transparent on candling than "floating yolk" eggs.

The investigations, the results of which are recorded here, were undertaken with the object of determining the nature of the "floating yolk" condition, and if possible to obtain information which would render elimination of the fault possible.

EXPERIMENTAL WORK.

As a preliminary investigation, a protein estimation was made (by Mr. R. J. Cowan, of the Government Laboratory of Bacteriology and Pathology) on the white of these eggs, with the object of determining whether the protein content in the white was lower than normal, as in the condition known as "watery white".

For this purpose three dozen eggs containing "floating yolks" were obtained, and the whites were thoroughly mixed. Micro-Kjeldahl estimations were then made, and the results compared with similar estimations carried out on the mixed whites of three dozen normal eggs.

Fig. 1. Contents of a "floating yolk" egg, showing thickened chalazae and the slightly opaque zone which surrounds yolk.

The factor 6·25 was used to convert the nitrogen percentage into protein percentage.

The results were as follows:

	Protein Content of Egg-white.
(i) Normal eggs	10·6 p.c.
(ii) Eggs with "floating yolk"	11·0 p.c.

The results of non-protein nitrogen estimations were identical in both cases. Determined by a colorimetric method, in each case it was approximately 25 mg. per 100 c.c. of egg-white. The small difference in protein percentage is no doubt within the limits of experimental error. The conclusion is that the protein content of "floating yolk" eggs did not differ from that of normal eggs, and in this respect the condition differed from that known as "watery white".

Changes Noted in Eggs with "Floating Yolks".

Besides the characteristic "floating" condition of the yolk, which could always be readily demonstrated by candling, the shadow of what appeared to be an appendage was sometimes plainly visible. Usually, when such appendages were visible, only one was present, but in some eggs two such structures were seen.

When the eggs were broken, there was usually little apparent change. In many instances the yolks appeared normal in colour. In others, there was a definite darkening of the yolk colour, which in many cases was associated with a partial coagulation of the proteins, giving the yolk a "candied-honey" or "lemon-cheese" appearance. In a few cases, besides this partial coagulation of the main mass of the yolk, small plaques of dried, or inspissated yolk were found adherent to the vitelline membrane.

Fig. 2. Contents of a "floating yolk" egg, showing thickened chalaza on right and normal chalaza on left of yolk.

In a small percentage of eggs the yolk appeared a brownish-green colour, and in these cases there was an associated partial liquefaction of the yolk.

In the great majority of these eggs the white appeared slightly opaque, either throughout the whole of the white or, more frequently, in a zone immediately surrounding the yolk (Fig. 1).

The most striking change, however—and one which was present in all affected eggs—was noticed in connection with the chalaza, or suspensory ligament of the yolk. One, or sometimes both, showed considerable enlargement with marked increase in opacity of the egg-white immediately surrounding it (Figs. 1 and 2). The normal spiral arrangement, if still present, was less marked than normal, but in most cases it was absent. The distal end (the end nearest the shell) was always ragged in appearance, and showed signs of partial digestion. By observing the way in which the free yolk travelled in candling the egg, and, when possible, noting the movements of the shadow cast by the thickened chalaza, it became fairly clear that this thickened condition of the chalaza was probably related to the "floating yolk" condition in the nature of cause and effect. The remainder of the observations here recorded are the results of investigations intended to elucidate the nature of this pathological change in the chalaza.

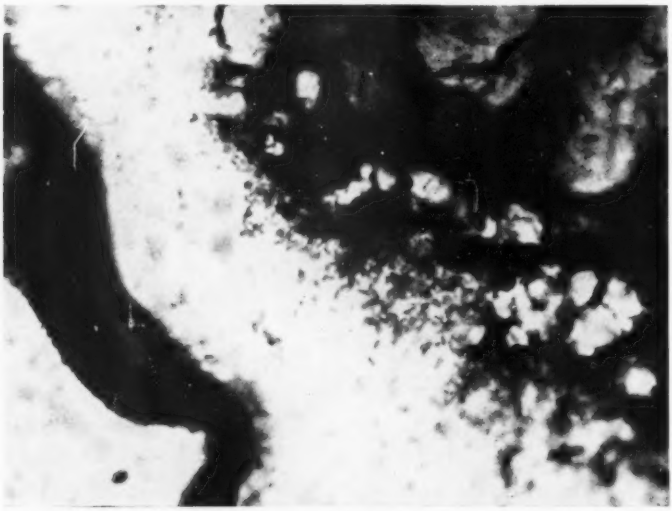

Fig. 3. Transverse section of thickened chalaza from "floating yolk" egg, showing masses of organisms present in interior of ligament. (× 1200.)

Results of Microscopic Examination of Paraffin Sections of Thickened Chalaza.

Most of the section appeared quite structureless, and probably consisted of coagulated egg-white. In sections suitably cut so as to pass transversely through the chalaza, the structure of this organ was easily seen. The ligament itself was considerably thickened, but all detail was lost.

When examined under high power, deeply-stained masses of what appeared to be bacteria could be seen enclosed in the hollow interior of the chalaza. Examination under a $\frac{1}{12}$ in. oil-immersion confirmed this finding. In sections stained by Gram's method, the organisms appeared rod-shaped, with rounded ends which in some of the organisms were somewhat club-shaped. The Gram stain was retained (Fig. 3).

The Isolation and Cultivation of the Organism.

In attempting to isolate this organism, cultures were made from the white and yolk of the egg, using sterile Pasteur pipettes to take up the inoculum. Cultures were incubated at 37° C. for several days under both aerobic and anaerobic conditions. In most cases the results were negative. When, however, the affected chalaza was ground up in a sterile tube with the aid of a glass rod, and cultures inoculated from the ground-up material, successful results were obtained in almost every case.

It is interesting to note here that if some of the ground-up chalaza were diluted with sterile saline and examined microscopically as a hanging drop preparation, motile rod-shaped organisms were seen in large numbers, occurring either singly or in short chains of two or three individuals. The most striking feature was the extreme rapidity of the movement of these organisms.

The Morphology and Staining of the Organisms isolated from the Chalaza.

In films prepared from twenty-four hour cultures on solid media, the organisms appear singly and in chains of two, three, or more. They are rod-shaped, with parallel sides and rounded ends, and are on an average 3 μ long and 0·75 to 1·0 μ wide. In older cultures spores are produced. These are oval in shape, the longitudinal diameter being nearly twice the transverse diameter, and are situated subterminally, where they produce some bulging of the organisms. Sporing appears to take place readily. In cultures incubated for longer than twenty-four hours, or even left at room temperature for one or two days following twenty-four hours' incubation, most of the organisms appear in the sporing form. In broth cultures the organism is actively motile. A capsule has not been demonstrated.

In films from young cultures the bacillus stains readily with the usual aniline dyes, and is Gram-positive. The spores usually appear unstained, except when special methods are used. In older cultures staining is not so even; many shadow forms appear, and many of the organisms tend to lose the violet stain in Gram's method if the decolourization process is continued too long.

Cultural Characteristics.

The most characteristic type of growth is seen when the organism is grown on blood agar plates and in broth.

In blood agar plate cultures twenty-four hours old an irregular type of colony is produced. It is lobulate in structure, with the number of lobes varying between two and four. Each lobe has a raised dome-shaped centre and an irregular edge, and the point at which all the lobes of the colony join is usually lower than the apex of the constituent lobes. In cultures between 18 and 24 hours old the surface of the colony is smooth and glistening, and at intervals along the margin of the lobes small beads of moisture are scattered. In colour the colonies are greyish-white, and are quite opaque. The growth is readily emulsified at this stage. When a colony is touched with a platinum wire it collapses in the same manner as a watery bleb of the skin when punctured with a needle. In 18 to 24 hour cultures the colonies appear to consist of a thin pellicle stretched over a drop of moisture, and attached firmly to the media at the margin (Fig. 4).

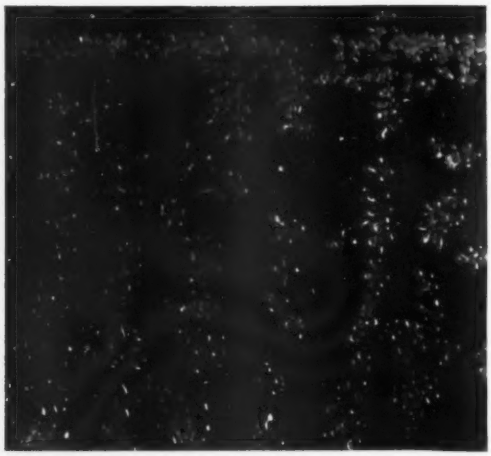

Fig. 4. A 24-hour culture of the bacillus of blood agar medium. (× 1·25.)

In cultures more than 30 hours old this appearance is lost, and each colony appears dry, wrinkled, and quite flat, although maintaining the same characteristic lobate arrangement.

On agar slopes a confluent growth results, but the characteristic appearance of the growth in early plate cultures is not seen. The surface is dry and wrinkled, and the margin is irregular and often rhizoid in appearance.

In broth cultures growth takes place on the surface only, and a tough, wrinkled pellicle is formed within eighteen hours. The surface of the pellet is dotted with small glistening globules of moisture, which often remain for several days, especially if excessive evaporation is prevented.

In broth cultures two or three days old there is usually a complete absence of turbidity. After five days' incubation a membranous deposit makes its appearance, and is augmented in amount chiefly by additions from the pellicle. About this time also the broth becomes turbid, but the turbidity produced is never very marked, being approximately equivalent to that produced by 200 million organisms per c.c

In gelatin stab cultures incubated at 22° C. no growth is visible until after at least 72 hours' incubation, and then the colonies are only 0·5 mm. in diameter. With longer incubation very little increase in size of the colonies occurs, but by the seventh day liquefaction of the stratiform type is observed.

When the gelatin culture is incubated at 37° C., small fluffy colonies about 1–2 mm. in diameter make their appearance throughout the stab in less than 24 hours. After 72 hours' incubation at 37° C. the whole of the gelatin has been changed, so that it remains permanently liquid, even at temperatures below that at which melted gelatin normally solidifies.

With regard to the metabolism of the organism, a few points are of interest.

It is markedly aerobic in its oxygen requirements, and it will not grow in media containing a reducing substance or in an anaerobic jar. It grows readily on ordinary laboratory media, and growth is not increased by the addition of serum, blood, or glucose. On blood agar media a zone of haemolysis of the β-type is produced.

Although no pigment is produced on solid media, when broth cultures are left in the dark for longer than a week the pellicle becomes brick-red in colour.

Post-mortem Examination of Hens laying Eggs with "Floating Yolks".

Four hens were selected at random from among a batch of birds which were laying eggs with "floating yolks" for post-mortem examination. No abnormality could be detected in any of these hens. Sections of portions of the oviducts were examined, but no lesion of any kind was seen.

With three of the birds, cultures were made from several regions of the oviduct and from a large number of the developing ova in the ovary. In all cases cultures from the ovaries were sterile. From the oviduct of one bird positive cultures were obtained. In a tube of broth inoculated with material scraped from the mucous membrane of the upper inch of the oviduct, a pure culture of the organism already described was obtained. Cultures from the lower end of the oviduct were mixed, and contained principally Gram-positive cocci and Gram-negative bacilli. Owing to the fact that the bacillus already isolated from "floating yolk" eggs had been recovered in pure culture from the upper end of the oviduct, no attempt was made to determine its presence in the mixed cultures obtained from the lower end of the duct.

Attempts to produce the "Floating Yolk" condition in Normal Eggs by infecting Healthy Birds with the Bacillus.

Three separate experiments were performed, two at the Government Poultry Farm at Parafield, and the third at the Government Laboratory of Bacteriology and Pathology. Great difficulty was experienced in obtaining birds which were not laying "floating yolk" eggs, and consequently only twelve birds were available. These birds were placed in separate pens, which were sterilized as effectively as possible. The pens used at Parafield were of wood and wire netting, and hence could not be sterilized as adequately as those used at the Laboratory, which were made of galvanized iron and wire netting.

All the birds had been under observation for periods ranging from one to three weeks, during which no birds had laid a "floating yolk" egg. During the course of the experiment they were fed on whole wheat, which had been autoclaved for 20 minutes at a temperature of 115° C. and a pressure of 15 lb. above atmospheric pressure. In addition to the whole wheat, they were given crushed wheat prepared from the sterilized grain in a crusher sterilized before use, and kept solely for that purpose. To this was added lucerne, which had been grown locally. Drinking water was obtained from the main supply or from tanks.

In each experiment spores of the bacillus, obtained by washing off a five-day agar plate culture in normal saline, were added to the drinking water so as to give a concentration of about 50 million spores per c.c. of water. In addition, each bird was given, each day, one pellet of crushed meal, moulded with the aid of a little water to which spores had been added. This treatment was continued for five successive days.

In the three experiments all birds commenced to lay "floating yolk" eggs within periods ranging from two days to three weeks. In birds laying regularly at almost daily intervals the condition appeared in the second or third egg laid after commencement of the experiment. Several of the birds were laying only at irregular intervals, and in two cases 16 and 22 days respectively elapsed between the commencement of the experiment and the laying of an egg. In these cases the first egg showed the "floating yolk" condition.

A number of the eggs laid by the birds used in the three experiments were examined and cultured. The typical lesions were present, and the characteristic bacillus was recovered from several eggs.

DISCUSSION.

On its morphology and metabolic requirements the organism recovered from the affected eggs must be placed in the Genus Bacillus, but whether it corresponds with any of the species so far named is not quite certain.

Comparison with a large enough number of species of the Genus Bacillus has not yet been made, and until such has been done it is not permissible to give this

organism a species name. When time permits, an attempt will be made to classify it fully.

Its presence in the affected eggs is of considerable interest, but whether it is related to the condition known as "floating yolk" as the primary causal agent, or only secondarily, has yet to be determined. The frequency with which it can be recovered from affected eggs, its presence in the oviduct of birds laying "floating yolks", and its known property of being able to liquefy protein, suggest that it is connected with the condition, and the feeding experiments also point in that direction. The small number of birds used, 12 in all, and the absence of adequate controls, make one hesitant to draw conclusions, despite the fact that in each experiment all birds started laying eggs with "floating yolks" within reasonable periods of time after the commencement of the experiment, while previously they had laid normal eggs. The extreme difficulty in obtaining normal birds prevented any more adequate test being carried out.

In the light of the observations already made, we might postulate an hypothesis as to the means by which the organism gains entrance to the egg, and the changes which it produces or assists in producing there.

The organism is probably taken into the alimentary tract, and, after passing through the ventricula, either in the spore or vegetative form, reaches the cloaca, whence it passes up the oviduct. In the upper third of the duct it is in a position to become involved in the chalaza before the white is added to the yolk. In the chalaza it multiplies, and as the result of its metabolic processes, the chalaza undergoes partial liquefaction and ruptures, allowing the yolk to float freely.

It is unlikely that the birds themselves become infected in the strictest sense, as they always appear perfectly healthy. It must be admitted, however, that the organism is non-pathogenic. Mice are able to tolerate 2 c.c. of a 24-hours' broth culture intraperitoneally, and hens show no local or general reaction following intramuscular injections of 2·5 c.c. of the same culture.

Further observations are being made in order to elucidate the relationship of this organism to the "floating yolk" condition, and also into the possibility of treatment by means of vaccines, and these will be presented in a communication at some future date.

SUMMARY.

1. A condition of fowl eggs termed "floating yolk" is described. The nature of the changes present in the egg and the distribution of the condition are discussed.

2. An aerobic spore-bearing Gram-positive bacillus, which can be isolated from these eggs, is described.

3. Experiments in which birds that had previously laid normal eggs commenced to lay "floating yolk" eggs when fed with spores of the bacillus are described, and the results discussed.

4. The probable rôle of this bacillus is discussed.

ACKNOWLEDGMENTS.

In conclusion the author desires to thank all those who have helped him in any way, especially Mr. Anderson, for assistance in the feeding experiments; Mr. Cowan, who carried out the biochemical investigations, and Mr. Tee, who took the photographs with which this paper is illustrated.

REFERENCES.

Anderson, C. F. (1936): Bull. Dept. Agric., S.A., No. 308.
Halnan, E. T. and Day, H. D. (1935): J. Min. Agric. (England), 42, p. 236.

A NEW METHOD FOR DETERMINING THE CENTRE OF GRAVITY OF THE HUMAN BODY

by

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(Submitted for publication 28th March, 1936.)

INTRODUCTION.

In 1906 Irving Fisher published a method for indicating food values. In this article an apparatus was described for rapidly determining the centre of gravity of a small triangular card weighted at various points with pins of several dimensions. The card was placed in a basket and suspended on a standard so that the centre of gravity of the pin system assumed a position vertically under the point of support. By adjustment of counter-weights the centre of gravity of basket plus unweighted card coincided with the point of support, so that the system was in neutral equilibrium.

When I obtained the apparatus, I thought at once of using a similar device for determining the centre of gravity of the human body, but soon realized that, as the weight of the moving system would be so enormously greater than the light card described, something more was required than merely magnifying the dimensions. In fact the problem required engineering experience for its solution. Many years passed before I took up the subject again, and then I made the suggestion to Mr. A. H. Faul, Lecturer in Graphics and Engineering Design in Melbourne University. With the permission and co-operation of Professor Kernot, to whom our thanks are extended, Mr. Faul designed and constructed the instrument now described, and to him is therefore due the success of the method. W.A.O.

DESCRIPTION OF THE APPARATUS.

For convenience in describing the apparatus a division has been made into four separate sections, which will be taken in the following order:

(a) Moving System;
(b) Supporting System;
(c) Damping System;
(d) Indicating System.

(a) The Moving System consists of a timber table A, 7 ft. x 3 ft., on which the subject is placed in any desired position. Two short extensions are provided for the subject's arms when lying with the arms extended sideways.

This table is supported by four rods to the part C, which contains four bearings arranged in a gimbal, to allow the table to swing about this point in either direction. Small self-aligning ball bearings, size 4 mm. shaft, are used for the parts of the gimbal. These give a very satisfactorily high degree of sensitivity.

Apparatus as set up in the Physiological Department, Melbourne University.

Fixed rigidly to the table by means of the vertical rod above C and the long bracing rods, is a set of balance weights B. The positions of these may be varied by screwing them along the cross bars, and by this means the whole moving system may be brought into neutral equilibrium before the subject is placed on the table. A ladder is provided at G, also a platform with handrail to facilitate adjusting the weights B.

The degree of movement of the table provided is approximately 6 in. in each direction.

(b) Supporting System: A steel frame F in the form of a triangle is mounted on three levelling screws which rest on plates on the floor. This triangular frame carries two cross beams, to which are bolted the large vertical standards G. These are braced together, and carry beams at their top ends which give the support to the bearings of the gimbal C, and thus support the moving system.

(c) Damping System: To bring the table more quickly to rest in its equilibrium position when the subject is lying on the table, a damping system is provided. This consists of four rectangular water-tanks H fixed to the base F. Four flat steel plates, two across the table, are attached to the underside of the table, and dip into the water in these tanks. There is one plate in each tank, and each plate has a series of small holes drilled through it to increase the damping effect.

(d) Indicating System: It has been previously pointed out that before the subject is placed on the table the whole moving system is brought into neutral equilibrium. Thus when the subject is placed on the table the moving system will swing about the gimbal support C, until the centre of gravity of the *subject alone* will lie under the centre point of the gimbal.

A plumb-bob D is supported from a point of the gimbal, and this may be lowered on to the subject to indicate the exact position of its centre of gravity.

If, instead of finding the actual position of the centre of gravity, it is desired to measure the change in the position of the centre of gravity, when say breathing takes place or a limb is moved to a different position, a table E is provided for the differential readings. This table has attached to it a piece of squared paper. A pointer consisting of two lines scribed on a piece of celluloid and fixed to the table A indicates the table movement on the paper at E. Readings to ·01 in. can reasonably be taken on this pointer.

The movement of the table is limited by means of an iron pipe secured to the underside of the end passing through a square hole cut in a plate which is fastened to the base. This pipe cannot be seen in the photograph, as it is located between the four damping tanks H.

A clamping device is also provided, operating on the pipe mentioned above, and is located just under the damping tanks. The clamp is operated by a hand lever, the top of which can just be seen to the left of the letter E in the photograph. The clamp is simply to save the apparatus from damage when the subject is climbing on to or off the table.

It was found that when the moving system was adjusted to neutral equilibrium a mass of only one gramme, placed 25 cm. from the centre of the table, completely upset the balance, which shows that the ball bearing support gave a very satisfactory arrangement, considering that the moving system weighs approximately 250 lb.

REFERENCE.

Fisher, Irving (1906): Amer. J. Physiol., 15, p. 417.

THE ABSORPTION SPECTRA OF SOME MODIFIED SNAKE VENOMS

by

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(From The Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 14th April, 1936.)

Experiments have been carried out in this Institute with a view to the preparation from snake venoms of substances possessing a more limited range of physiological effects than the original venoms. From the venom of *Notechis scutatus* and of its melanotic variant substances were prepared (Holden, 1933) which had practically no coagulant action on blood, but which retained the other toxic powers of these venoms. A somewhat similar separation was effected in the case of the venom of *Acanthopis antarcticus* by extraction of the venom with an aqueous solution containing 45 p.c. of ethyl alcohol (Kellaway, *et al.*, 1929).

TABLE 1.

Maxima and Minima of the Absorption Spectra of Modified Venoms.

	Maximum $\nu 10^{-12}$	ϵ	Minimum $\nu 10^{-12}$	ϵ
Notechis scutatus venom	1135	2·30	1215	2·00
" " "neurotoxin"	1147	3·00	1215	2·30
Notechis scutatis var. niger venom	1123	1·75	1210	1·40
" " " "neurotoxin"	1147	2·90	1209	2·64
Acanthopis antarcticus venom	1143	3·15	1217	2·65
Fraction soluble in 45% C_2H_5OH	1149	4·50	1224	3·20
Fraction insoluble in 45% C_2H_5OH			no band.	

ν = frequency

$$\epsilon = \frac{1}{cd} \log \frac{I_0}{I}$$

c = number of grams in 1 litre
d = depth of liquid in cm.
I_0 = intensity of entering beam
I = intensity of emergent beam.

In this paper are recorded the ultra-violet absorption curves of these derivatives. In addition a study has been made of the effects on the absorption curves and the haemolytic powers of the venoms of *Denisonia superba* and *Pseudechis porphyriacus* of treatment by heat and by formalin. The determination of the

TABLE 2.

The Inactivation of Haemolysins by Heat.

		Venom.	Water.	Acid.	Alkali.	Time.	Temperature.	Percentage Active pH 6·0	Maximum $\nu 10^{-12}$	ϵ	Minimum $\nu 10^{-12}$	ϵ
A.	*Pseudechis porphyriacus*											
	1.	5·0 mg.	0·5 c.c.	0·2 c.c. N.HCl	—	2 hours	59°C.	38	1075	1·32	1181	·60
	2.	5·0 mg.	0·5 c.c.	0·5 c.c. N.HCl	—	2 hours	59°C.	23	1073	1·32	1183	·66
	3.	5·0 mg.	0·5 c.c.	—	0·2 c.c. 0·02N.NaOH	2 hours	59°C.	5	1083	1·74	1161	1·40
	4.	5·0 mg.	0·5 c.c.	—	1·0 c.c. 0·02N.NaOH	2 hours	59°C.	1	No Band.			
	5.	untreated venom							1075	1·46	1193	·86
B.	*Denisonia superba*											
	1.	5·0 mg.	0·5 c.c.	0·2 c.c. 0·05N.HCl	—	2 hours	60°C.	100	1143	3·08	1217	2·52
	2.	5·0 mg.	0·5 c.c.	0·5 c.c. 0·05N.HCl	—	2 hours	60°C.	100	1145	3·20	1209	2·64
	3.	5·0 mg.	0·5 c.c.	1·0 c.c. 0·05N.HCl	—	2 hours	60°C.	100	1139	3·08	1215	2·40
	4.	5·0 mg.	0·5 c.c.	1·0 c.c. N.HCl	—	2 hours	60°C.	22	1142	3·08	1216	2·52
	5.	5·0 mg.	0·5 c.c.	—	1·0 c.c. 0·05N.NaOH	1·6 hours	37°C.	2	No Band.			
	6.	10·0 mg.	1·0 c.c.	—	0·5 c.c. 0·05N.NaOH	1·6 hours	60°C.	0	1156	3·50	1200	3·00
	7.	untreated venom							1147	3·08	1222	2·64

TABLE 3.

Inactivation of Haemolysins by Formalin at 18-25° C.

		Venom mg. per c.c.	Formalin per cent.	Time-days.	Percentage active haemolysin remaining. pH 6·0	pH 7·7	Maxima $\nu 10^{-12}$	ϵ	Minima $\nu 10^{-12}$	ϵ
C.	*Pseudechis porphyriacus*									
	1.	5	0·4	1	82	58	1069	1·43	1188	·80
	2.	5	0·4	3	58	34	1069	1·46	1200	·70
	3.	5	0·4	10	23	17	1073	1·46	1173	·86
	4.	untreated venom					1075	1·40	1188	·80
D.	*Denisonia superba*									
	1.	5	0·2	1	66	50	1128	3·50	1204	2·80
	2.	5	0·2	2	—	—	1126	3·85	1200	3·36
	3.	5	0·2	5	—	—	No Band.			
	4.	5	0·2	8	23	15	—	—	—	—
	5.	untreated venom					1134	2·92	1209	2·40

A different specimen of venom of *Denisonia superba* was used for inactivation by formalin from that inactivated by heat.

absorption curves was done as described in a previous paper (Holden and Setter, 1935). The amount of haemolysin remaining active was determined from the logarithmic time-concentration curves of the experiments, which were all carried out exactly as described in previous papers on haemolysis by snake venoms (Holden, 1934 and 1935). The determinations of the residual haemolytic activity were made at about ten different concentrations over as wide a range as possible. The haemolytic activity of the venoms treated with formalin was determined both at pH 6·0 and at pH 7·7 in the presence of lecithin. There appeared to be consistently somewhat less remaining activity according to the latter method of determination.

The venoms were heated in sealed tubes in a thermostat under the conditions stated in the tables. After being cooled the solutions were neutralized and their haemolytic power determined. A sample was retained for determination of the absorption spectrum. In acid solution both haemolysins were fairly resistant to heat at 60° C. In alkaline solution inactivation was very rapid. In acid solution little change was observed in the absorption spectrum, even when most of the haemolysin had been inactivated. In alkaline solution the maxima and minima rapidly increased, and finally the band was obliterated. Both haemolysins were inactivated by formalin, that of *Pseudechis porphyriacus* being rather more resistant. In the case of the venom of this snake there was practically no change observed in the absorption spectrum due to the action of formalin, while with the other venom a lower concentration of this reagent entirely obliterated the band in a few days.

DISCUSSION.

It has often been suggested that the absorption band which is present in the ultra-violet absorption spectra of many proteins is to be attributed to their content of amino-acids with a benzene ring. Snake venoms appear to be proteins, and this explanation would presumably apply to them also. It is, however, somewhat difficult to accept this view altogether, owing to the marked changes in the band brought about by reactions, e.g. heating in very dilute alkali, which do not attack any of the aromatic amino-acids, even tryptophane. It would appear that the band may be associated with those unknown constitutional elements which are related to the high solubility of native proteins and to the physiological activities of venoms. It would also seem probable that the reactions of inactivation by acid and by alkali of the haemolysins involve different structural elements of the molecules, in addition to those actually involved in the haemolytic activity. The difference observed in the degree of inactivation according to whether the activity was measured in acid solution or in alkaline solution with lecithin, would appear to indicate two types of haemolytic factor. One attacks the cell directly; the other converts lecithin into a chemical lysin.

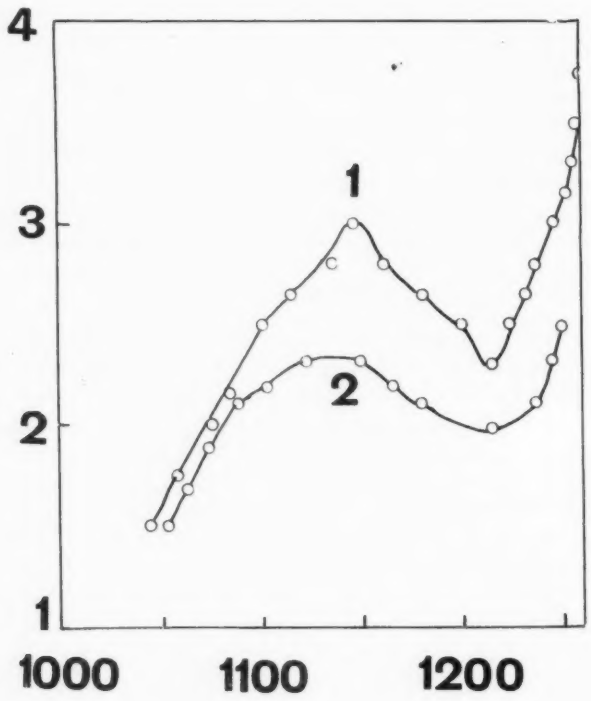
The author is indebted to Professor W. A. Osborne, of the University of Melbourne, for the loan of the spectrophotometer used in these experiments.

SUMMARY.

The changes in the absorption spectra of snake venoms of which the haemolysins have been inactivated by heat in acid or alkaline solution, or by formalin, are described. The absorption spectra of certain fractionated venoms are recorded.

REFERENCES.

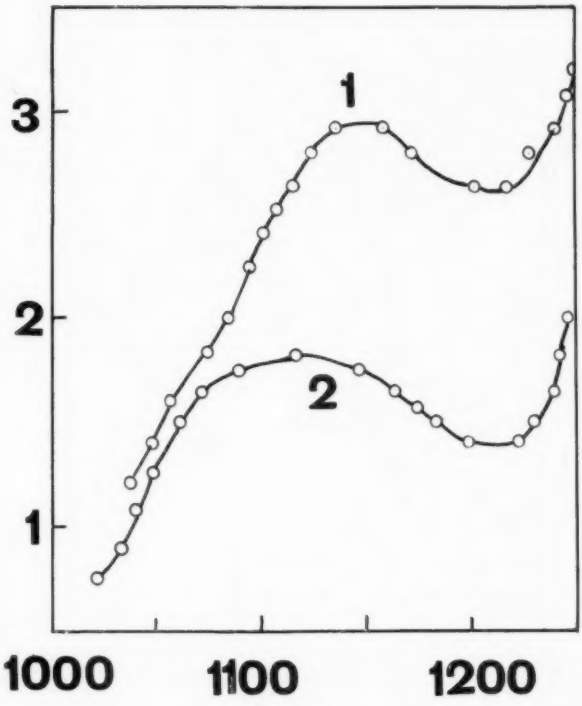
Holden, H. F. (1933): Austral. J. exp. Biol., 11, p. 1.
Holden, H. F. (1934): Austral. J. exp. Biol., 12, p. 55.
Holden, H. F. (1935): Austral. J. exp. Biol., 13, p. 103.
Holden, H. F. and Setter, C. G. (1935): Austral. J. exp. Biol., 13, p. 223.
Kellaway, C. H., Freeman, M. and Williams, F. E. (1929): Austral. J. exp. Biol., 6, p. 245.



Graph 1.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$

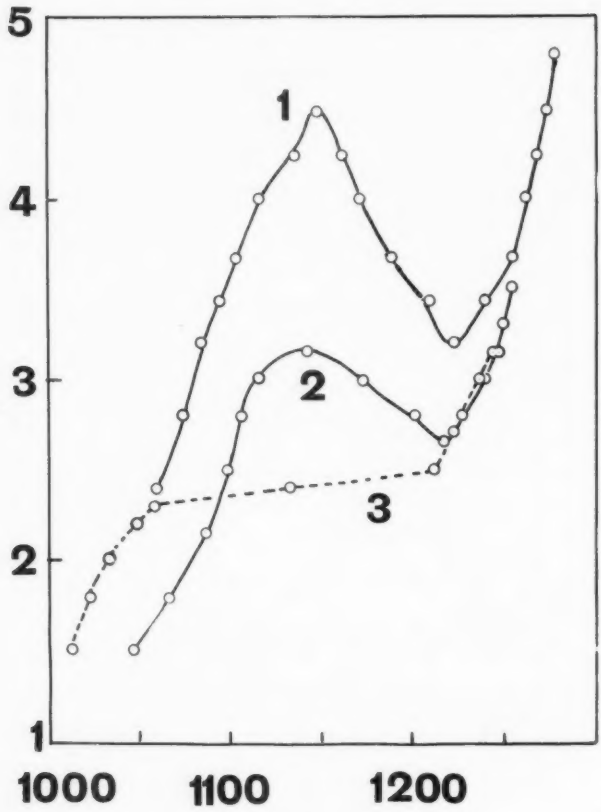
Curve 1. "Neurotoxic" fraction of *Notechis scutatus*.
" 2. Venom of *Notechis scutatus*.



Graph 2.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$

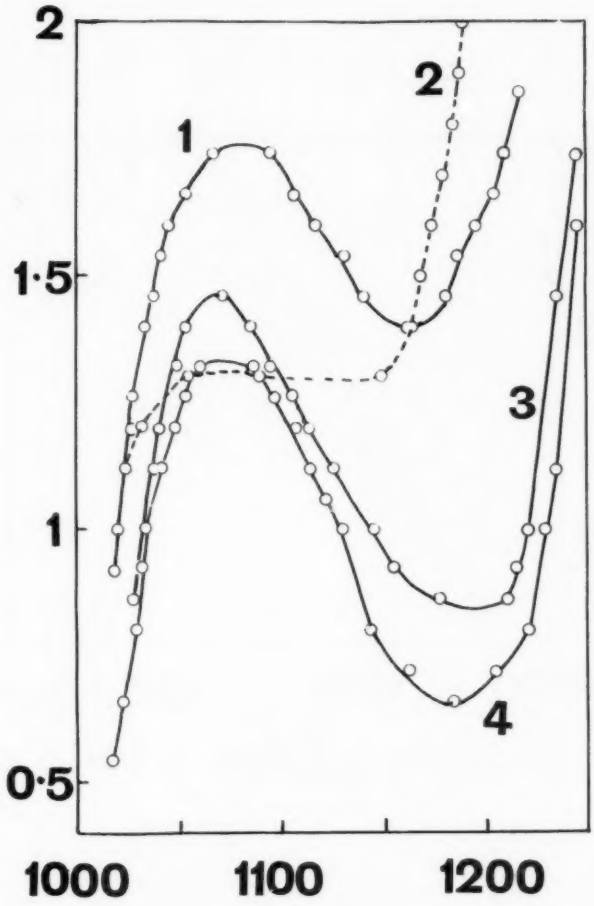
Curve 1. "Neurotoxic" fraction of *Notechis scutatus var. niger.*
„ 2. Venom of *Notechis scutatus var. niger.*



Graph 3.

Ordinates $= \epsilon$
Abscissae $= \nu 10^{-12}$

Curve 1. Fraction of venom of *Acanthopis antarcticus* soluble in 45 p.c. ethyl alcohol.
,, 2. Venom of *Acanthopis antarcticus*.
,, 3. Fraction of venom of *Acanthopis antarcticus* insoluble in 45 p.c. ethyl alcohol.

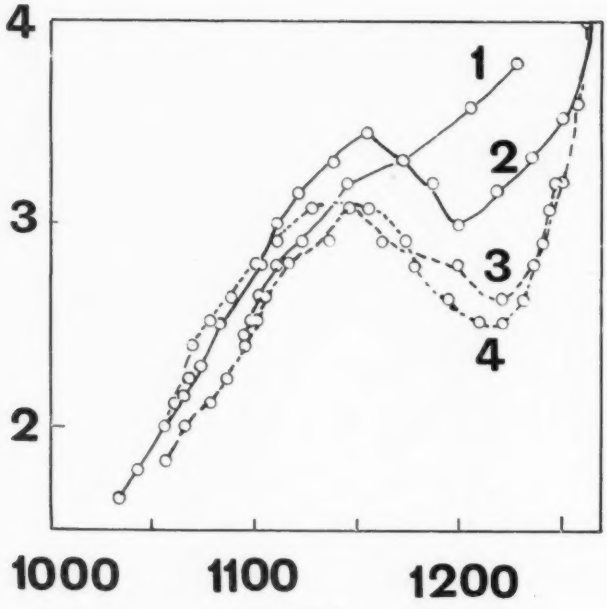


Graph 4.

The effect of heat on the venom of *Pseudechis porphyriacus*.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$

Curve 1 = Table 2A, No. 3. Heated alkaline.
„ 2 = „ „ No. 4. Heated alkaline.
„ 3 = „ „ No. 5. Untreated venom.
„ 4 = „ „ No. 2. Heated acid.

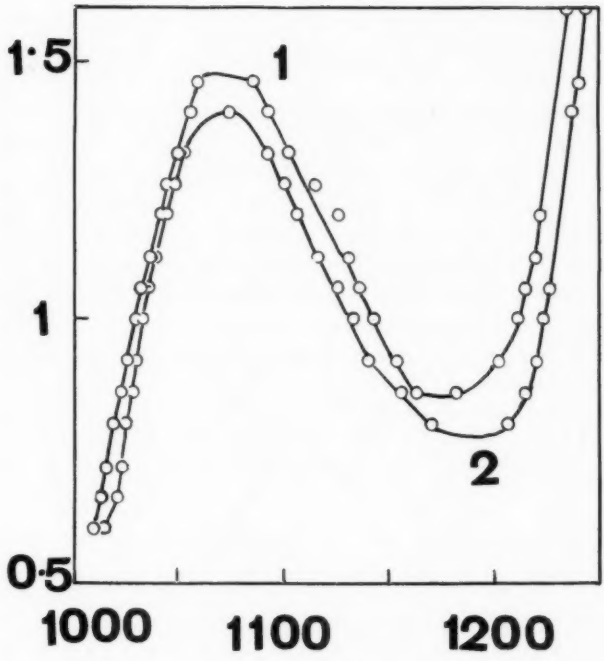


Graph 5.

The effect of heat on the venom of *Denisonia superba*.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$

Curve 1 = Table 2B, No. 5. Heated alkaline.
„ 2 = „ „ No. 6. Heated alkaline.
„ 3 = „ „ No. 7. Untreated.
„ 4 = „ „ No. 4. Heated acid.

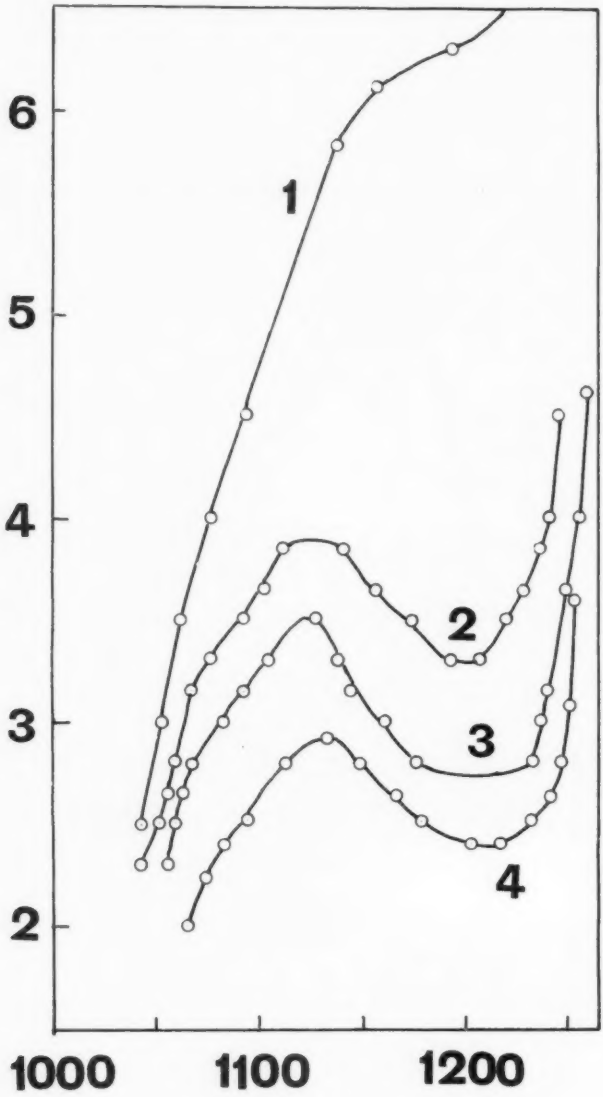


Graph 6.

The effect of formalin on the venom of *Pseudechis porphyriacus*.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$

Curve 1. Table 3C, No. 3. 10 days' formalin.
" 2. " " No. 4. Untreated venom.



Graph 7.

The effect of formalin on the venom of *Denisonia superba*.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$

Curve 1. Table 3D, No. 3. 5 days' formalin.
„ 2. „ „ No. 2. 2 days' formalin.
„ 3. „ „ No. 1. 1 day's formalin.
„ 4. „ „ No. 5. Untreated venom.

THE MECHANISM OF THE ACTION OF ETHYLENE ON CELL PROCESSES

by

F. F. NORD

(Department of Veterinary Physiology of the University, Berlin).

(Submitted for publication 26th February, 1936.)

The Australian Journal of Experimental Biology and Medical Science published a paper under the above title in volume 13, pp. 95-102 (1935), by F. H. Shaw, the work being carried out at the suggestion and under the supervision of Professor W. J. Young, of Melbourne. The paper consisted mainly of an attack on certain investigations of the author and his collaborators made during 1926-1930.

Since this journal is not very widely distributed in Germany, the paper was only noticed in the form of an abstract in the Berichte über die gesamte Physiologie, vol. 89, p. 621, appearing on January 8, 1936. Since no reprint of the original paper was sent to me, I obtained a photo-copy on January 14, 1936, and wish to submit the following remarks.

1. A preliminary communication of the first two papers of this series was published in Nature, vol. 120, p. 82, 1927 (dated April 12). The paper itself was read before the Imperial University, Kyoto, on September 28, 1927 (compare Protoplasma, vol. 4, p. 547, 1928). I therefore regard myself and not Harvey (1928), as suggested by Shaw, as responsible for the views concerning the influence of ethylene on the permeability of cells.

2. Nord and Franke, not Englis and Zannis (1930), were the first to employ enzymes prepared free from cell material for this work, the method employed being described in detail in Protoplasma, vol. 4, p. 557, 1928.

3. The experiments described on pp. 562-567 of the same paper make it quite clear that an increased fermentation with cell-free yeast juice was only noticed when the material had first been frozen and then thawed. This purely colloid-chemical transformation was not in any way connected with the ethylene effect. Nevertheless, it has been shown clearly that ethylene or acetylene when acting on the surface of the whole enzyme-carrier system slows down or extends the evolution of carbon dioxide when brought in contact with glucose (Z.f. Physiologische Chemie 183, p. 215), without precipitating the carrier system (Z.f. Physikalische Chemie A, 150, p. 16).

4. On p. 97 (last paragraph) Shaw states that he used for his measurements an apparatus (known to us) originated in 1911. We developed and described two different forms of apparatus in 1927 and 1929, which allowed simultaneous readings to be made every minute in parallel experiments. This enabled us to control the important initial phase of cell fermentation. I am at a loss to understand how the use of a rather primitive method justifies criticism of results obtained by our two improved methods. Moreover, when comparing our two methods (see Ergebnisse der Enzymforschung, vol. 1, p. 83, 1932) we gave numerical data concerning the accuracy of our blank experiments, and referred repeatedly to the degree of purity of our ethylene or acetylene used in the United States or in Germany. No such data, which are necessary for considering the speed of reversion of an adsorbed film, are available in Shaw's paper.

5. In our paper in the Journal of Biological Chemistry, vol. 79, p. 48, there are published curves which show clearly that the speed of fermentation with zymin (prepared by treating top yeast cells with acetone) can not be influenced by the presence of ethylene, due to the fact that the protein system governing cell permeability is destroyed.

6. Experiments carried out with tobacco leaf catalase (published in the above mentioned paper, p. 43) (see also the confirming results of Pfuetzer and Losch, Umschau, vol. 39, p. 204, 1935), with the enzyme system of a fermenting dough (Z.f. angewandte Chemie 32, p. 204, 1929), and with the enzymes of germinating barley (Z.f. Physiolog. Chemie 183, pp. 217-225, 1929), have given further evidence for the influence of alkylenes as well as acetylene on the permeability of cells.

7. Furthermore, in another paper (Protoplasma 11, 440, 1930), we have definitely proved that an increased penetration of dyes into the cells occurs when barley is steeped in water containing acetylene or ethylene.

8. The criticism in Shaw's paper (p. 101) concerning our carboxylase experiments is not valid, since the second apparatus enabling us to detect the details of the mechanism of this purely physical-chemical reaction was not yet constructed at that time.

9. The carrier theory of an enzyme system, originated by Mathews and Glenn, which is equally applicable within as outside the cell, shows that enzyme reactions are surface reactions. The influence of permeability on such a reaction, manifesting itself by increasing the reacting surface of the enzyme, is not difficult to understand, provided we do not expect—which would be quite incorrect—that this physiological regulation is retained in the so-called damaged, i.e. irreversible, phase. The effect lasts as long as ethylene or acetylene (in the case of acetylene the phenomenon is much more marked) is used in an appropriate manner; and the exosmosis from an ethylene-exposed yeast cell will be so small as compared with that obtained, for example, with toluene or chloroform, that our present methods presumably do not furnish us with an exact way of measuring it within the limits of accuracy. There is, therefore, no need, when explaining these gas effects, to

resort to the highly improbable conception of increased enzyme production by the cells, instead of accepting the interpretation of our experimental data as set forth in Science, vol. 79, p. 159, 1934.

10. The fact of adsorption of ethylene (compare Shaw, p. 101) on the surface of particles of yeast juices, as well as a great number of lyophilic colloids, has been proved. It has also been demonstrated with acetylene and nitrous oxide (see also F. A. Van der Laan, thesis, University of Utrecht, 1934). The changing degree of dispersion has been proved by a number of methods, and furnishes us with the means of understanding the increased activity of frozen zymase, tyrosinase, and peroxidase after thawing (Transactions Faraday Society 26, 760, 1930, Biochemische Zeitschrift 263, 353, 1933, Nature 135, 1,001, 1935.

11. A careful re-reading of our paper in the Z.f. Physiologische Chemie vol. 183, especially pp. 196-197, 206-207, 213-217, fails to disclose any foundation for the statement of Shaw in his discussion, that "Nord infers that ethylene, especially in the last stage, would be toxic". The opposite appears to be true according to the following quotation: "Die Möglichkeit, die Tätigkeit von Enzymlösungen durch diese Verbindungen fast vollkommen zu unterbinden, lässt den selbstverständlichen Schluss zu, dass auch in der lebendigen Zelle befindliche Enzyme eine Wirksamkeitsverminderung infolge Narkose erleiden können, ohne dass hierdurch eine Schadigung der Zelle angenommen werden müsste."

12. Reviews of the extensive experimental material indicated here were published in the Ergebnisse der Enzymforschung, vols. 1 and 2, where I had the collaboration of other Australian research workers (compare E. S. Horning: The enzymatic function of mitochondria, in vol. 2, 1933). I think that, before submitting our work to criticism, the authors concerned should have employed methods superior to those we have abandoned, and should have read more carefully at least those papers which were published in the English language.

Berlin, January 15, 1936.

PROFESSOR YOUNG'S REPLY.

The author of the paper criticized by Professor Nord (F. H. Shaw) is away from Australia, and, as the work was done in my laboratory, I wish to add a short note.

It was unfortunate that the suggestion as to increased permeability of cells as the result of exposure to ethylene was attributed to Harvey and not to Nord. This resulted from the fact that the first publication by Nord was made in Nature and in Protoplasma, and not in the usual scientific journals publishing original work.

Without entering into any argument, I might state that the method employed by Shaw for measuring the rate of action was capable of giving a rate in parallel

experiments every five minutes, which would appear of sufficient accuracy to show any marked effect produced by the addition of ethylene.

The work was not confined to the action on yeast cells, several other enzymes being also employed.

The ethylene used was a product sold for anaesthesia, and in all cases it was well washed before using.

W. J. YOUNG.

13th March, 1936.